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NITRIC OXIDE IN BRAIN CONTUSION

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To my family

ABSTRACT

Trauma to the brain is a leading cause of death and disability. The primary injury initiates a cascade of biochemical reactions in the brain that causes further neuronal damage, a process referred to as secondary brain injury. The mechanisms for secondary brain injury are not fully understood and successful treatments for traumatic brain injury (TBI) still remain elusive. This study was undertaken to explore the pathophysiological role of nitric oxide (NO) in experimental and human brain contusion.

The temporal pattern and cellular sources of the three nitric oxide synthase (NOS) isoforms were examined following experimental contusion. The immunoreactivities for the constitutive NOS isoforms: eNOS and nNOS were increased early during the first hours after the trauma in endothelial cells and neurons, respectively. The non-constitutive inducible NOS isoform, iNOS, was induced later mainly in inflammatory cells with a peak one day after the trauma. Brain biopsies from patients undergoing surgery for cerebral contusions were examined. We found that iNOS was induced following human contusions and with a temporal concordant pattern to our experimental findings. The cellular sources of iNOS differed at different times after trauma. The majority were inflammatory cells during the first twelve hours, while neurons dominated between one and three days after trauma. The trauma models were considered relevant for experimental studies and treatment trials, since the findings in humans and rodents were similar.

Posttraumatic treatments with a non-selective NOS-inhibitor, a selective iNOS-inhibitor, or a free radical scavenger were performed All drugs separately reduced histopathological damage at 24 hours and 6 days post trauma. Formation of the neurotoxic substance peroxynitrite, synthesized by NO and superoxide, was also reduced by the drugs. The combination of a non-selective NOS-inhibitor and a free radical scavenger did not show additive neuroprotection. The findings supported that NO produced during the first days were detrimental to the injured brain and that an important mediator of NO-mediated toxicity was the formation of peroxynitrite.

Four drugs with different mechanism of action, directed against inflammatory-, free radical- or calcium-mediated toxicity were administrated and examined concerning histopathological damage and influence on iNOS. All four drugs showed some neuroprotective properties and all reduced iNOS induction.

The present study supported the hypothesis that iNOS induction and NO played important roles in the development of secondary injury following TBI and that NO may be detrimental to the injured brain, due to formation of peroxynitrite. Posttraumatic pharmacological modulation of NOS may improve the outcome.

ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to in the text by their roman numbers.

I Gahm C, Holmin S, Mathiesen T. (2000) Temporal profiles and cellular sources of three nitric oxide synthase isoforms in the brain after experimental contusion. *Neurosurgery 46, 169-177.*

II Gahm C, Holmin S, Mathiesen T. (2002) Nitric oxide synthase expression after human brain contusion. *Neurosurgery 50, 1319-1326.*

III Gahm C, Danilov A, Holmin S, Wiklund PN, Brundin L, Mathiesen T. (2005) Reduced neuronal injury after treatment with N^G-nitro-L-arginine methyl ester (L-NAME) or 2-sulfo-phenyl-N-*tert*-butyl nitrone (S-PBN) following experimental brain contusion. *Neurosurgery. In press.*

IV Gahm C, Holmin S, Wiklund PN, Brundin L, Mathiesen T. (2005) Neuroprotection by selective inhibition of inducible nitric oxide synthase after experimental brain contusion. *Submitted*

V Gahm C, Holmin S, Rudehill S, Mathiesen T. (2005) Neuronal degeneration and iNOS expression in experimental brain contusion following treatment with Colchicine, Dexamethasone, Tirilazad Mesylate or Nimodipine. *Submitted*

TABLE OF CONTENTS

ABBREVIATIONS

AMPA…….α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate
ABC…….avidin-biotin complex
BBB…….blood brain barrier
BSA…….bovine serum albumin
CNS…….central nervous system
CSF……..cerebrospinal fluid
CT……....computerized tomography
Cy3……...indocarbocyanine
DPX…….distrene 80, dibutyl phthalate xylene
eNOS……...endothelial nitric oxide synthase
FITC……fluorescein isothiocyanate
FJ…….Fluoro-Jade
GCS…….Glasgow coma scale
GFAP……...glial fibrillary acidic protein
GOS…….Glascow outcome scale
ICP……...intracranial pressure
IFN-γ……interferon-gamma
IL…….....interleukin
iNOS……inducible nitric oxide synthase
L-NAME……. N^G - nitro-L-arginine methyl ester
L-NIL…….. L-*N*-iminoethyl-lysine
LPS……...lipopolysaccharide
MAb…….....monoclonal antibody
mRNA……..messenger RNA
NeuN……neuronal nuclei
nNOS……...neuronal nitric oxide synthase
NMDA…….N-methyl- D-aspartate
NO……nitric oxide
NOS…….nitric oxide synthase
NT……nitrotyrosine
PAb……..polyclonal antibody
PBS……..phosphate-buffered saline
PMNC……..polymorphonuclear cell
RNOS……..reactive nitrogen oxide species
ROS…….reactive oxygen species
S-PBN…….2-sulfo-phenyl-N-*tert*-butyl nitrone
TBI……...traumatic brain injury
TdT……..terminal deoxynucleotidyl transferase
TNF-α……..tumor necrosis factor alpha
TUNEL……TdT-mediated dUTP- nick end labelling

INTRODUCTION

Trauma to the brain is a leading cause of death and disability. Motor vehicle accidents, falls, violence and sports cause the majority of brain injuries. The incidence for all closed head injuries admitted to hospitals in Sweden is conservatively estimated to be 200 cases per 100,000 populations. The incidences of brain injury are highest in the age ranges 15-24-years old and in the elderly (Kraus, 1987). A sizeable fraction of the survivors demonstrate significant long-term disability with even mild and moderate injuries (Becker et al., 1990), making this disease a major public health problem. These facts emphasize the necessity to develop therapies that improve survival and also provide good neurological recovery for a good quality of life. The primary injury initiates a cascade of biochemical reactions in the brain that causes further neuronal damage, a process referred to as secondary brain injury. The therapeutic strategies in neurointensive care today focus on limiting the secondary brain injury but successful treatments still remain elusive.

CLASSIFICATIONS OF TRAUMATIC BRAIN INJURY

With head trauma, mechanical deformation can lead to either vascular and/or parenchymal injuries. Traumatic brain injuries (TBI) constitute a heterogeneous group of disease and the pathology, pathophysiology and clinical course vary depending on the type of primary brain injury. The resulting pathology for vascular injuries depends on the anatomic localization and can present as epidural-, subdural-, subarachnoidal- or intracerebral haematomas. Parenchymal injuries are classified as contusions, lacerations or diffuse axonal injuries. The primary injury may also be defined by the type or pattern of injury caused: diffuse or focal. Focal injuries include focal cerebral contusion, traumatic intracerebral haematoma, epi- and subdural haematoma, whereas the diffuse injuries include cerebral concussion, traumatic subarachnoid haemorrhage and diffuse axonal injury. It is not uncommon that blunt head trauma results in a TBI with a combination of vascular, parenchymal, focal and diffuse types of injuries. The complexity of human TBI pathology contributes to the failure to develop effective pharmacological treatments; it challenges the clinician as well as the scientist.

Secondary vascular-related alterations may also accompany the primary head injury. The term ischemia-reperfusion injury describes a pathophysiological mechanism involved in a number of different diseases. Depending on the type of primary injury, ischemia may be focal or global. Once the brain is injured, even minor periods of ischemia can precipitate significant deterioration and affect the outcome (Chesnut et al., 1993).

CEREBRAL CONTUSIONS

Cerebral contusions are caused by blunt trauma or acceleration of the head resulting in a focal lesion consisting of parenchymal damage and micro haemorrhages around the brain capillaries. Contusions are not necessarily contact injuries. There are three main types of cerebral contusions: fracture contusion, coup contusion and the contrecoup contusion (Lindenberg, 1971). In addition, gliding contusions occur in high-energy trauma (Lindenberg, 1971). Fracture contusion and coup contusion are seen at the site of the impact in presence or absence of a skull

fracture, whereas contrecoup contusion refers to a cerebral contusion located diametrically opposite to the site of impact. The most common sites for focal cerebral contusions are the frontal and temporal lobes (Fig. 1). Vasogenic oedema is present in and around the contusion area and the centre of the contusion generally becomes necrotic. The clinical course of cerebral contusions may be dramatic. A delayed deterioration in neurological function has been reported since 1965 in patients with temporal contusions (McLaurin and Helmer, 1965) and has later been shown to be associated with a successive increase of the size of the lesion observed in serial CT scans (Mathiesen et al., 1995, Stein et al., 1993), leading to worsened outcome (Stein et al., 1993). Brain oedema, as a result of secondary brain injury, is thought to be partly responsible for the delayed deterioration in clinical neurological symptoms and can be observed as an increase of intracranial pressure (ICP) and may also be detected by CT. Even though several steps in the pathophysiological reactions involved in secondary brain injury have been elucidated, new research findings are needed for development of a pharmacological treatment in the future.

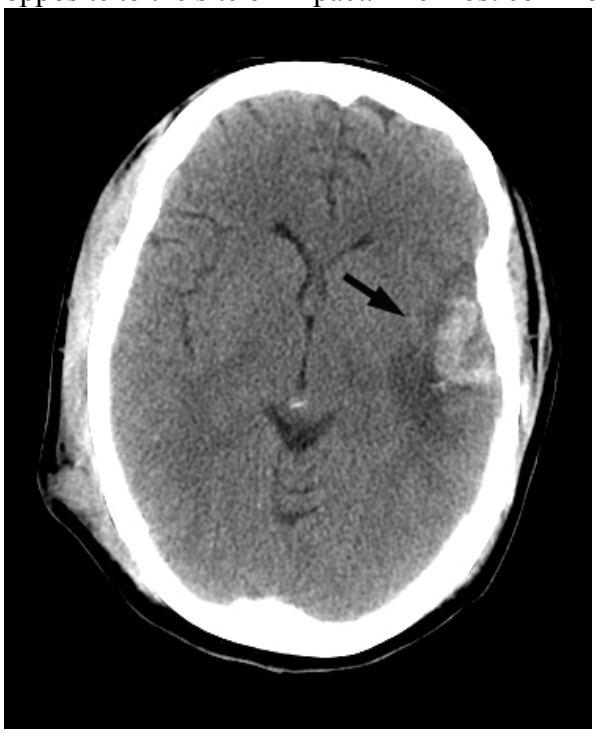

Figure 1 *CT-scan showing a temporal contusion. (light) with a central haemorrhagic component and a surrounding oedematous zone (dark), five days after a blunt trauma to the head.*

EXPERIMENTAL MODELS FOR CEREBRAL CONTUSIONS

From a clinical perspective, it is important to remember the heterogeneity of TBI. Thus, in experimental research several different models for contusional injuries have been used in a standardized way. The most relevant experimental models for contusional injuries are the cryogenic injury (CI) model (Clasen et al., 1953), the fluid percussion injury (FPI) model (McIntosh et al., 1989), the controlled cortical impact (CCI) model (Dixon et al., 1991) and the weight-drop injury model (WDI) (Feeney et al., 1981). All of them lead to a focal lesion in the brain parenchyma with different extents of hemorrhagic components and vasogenic oedema. In the CI model, a pre-cooled (liquid nitrogen) cylinder is placed on the dural surface of the brain causing a cold-lesion induced injury with minimal haemorrhage. The FPI model uses a transient fluid pressure pulse delivered to the brain to produce the injury. In the WDI model, a piston is dropped from a standardized height onto the exposed dura and underlying brain. In the WDI and the CCI models, depth of compression and energy transfer can be varied, which controls the severity of the injury. The WDI model causes a focal lesion resembling a cerebral contusion, with an expansion of the lesion, vasogenic oedema and a subsequent cavitation.

Another frequently used model of TBI is the closed head injury (CHI) technique (Nilsson B. et al., 1977), which produces a more diffuse injury, including diffuse axonal injury (DAI), by impact-acceleration-induction. One commonly used model of CHI was described by Marmarou et al. (Marmarou et al., 1994). This model produces a graded diffuse brain injury in the rodent

without a massive hypertensive surge or excessive brain-stem damage. The term DAI refers to an injury to the neuronal axons caused by the initial trauma-induced mechanical stretch and secondary injury biochemical reactions.

MECHANISMS OF SECONDARY BRAIN INJURY

The pathophysiological processes of secondary brain injury following a TBI are complex and incompletely understood. The excitotoxic damage, alterations in calcium homeostasis and free radical induced damage are thought to be key pathways. Moreover, the inflammatory response may be important in the exacerbation of secondary damage (Fig. 2).

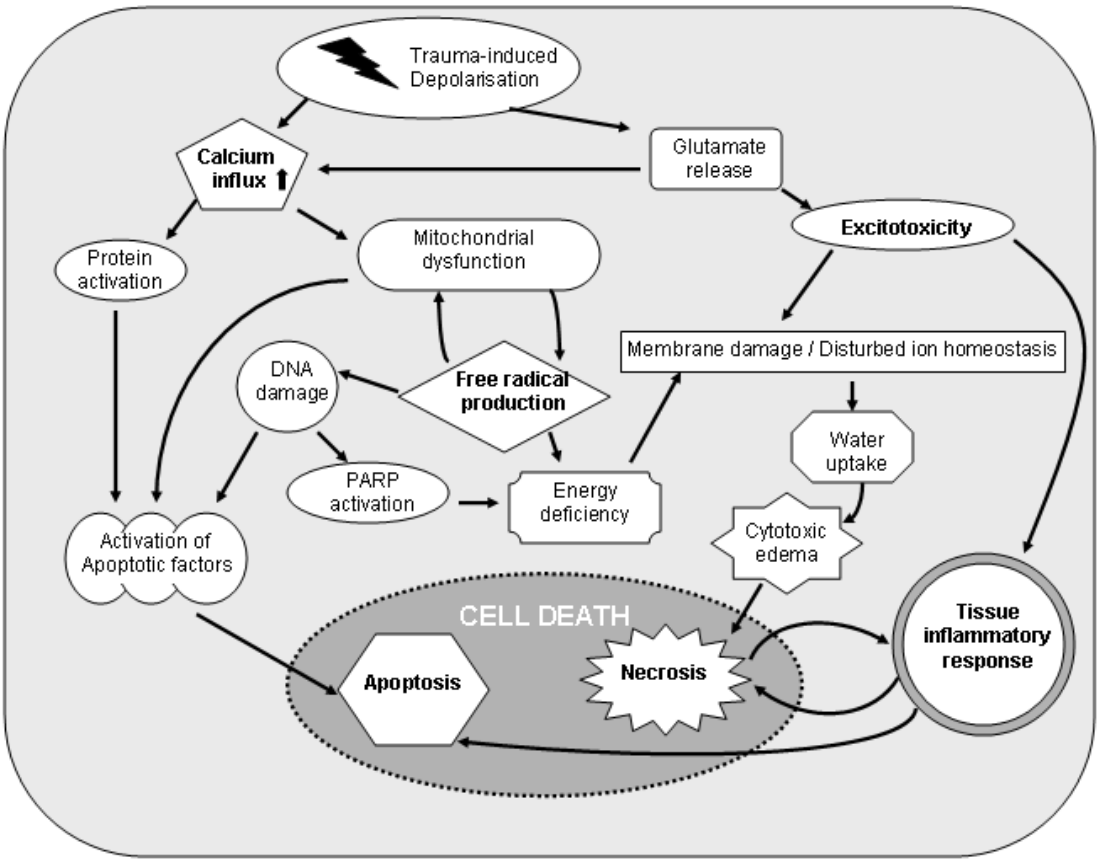

Figure 2. Biochemical reactions involved in secondary neuronal damage following TBI

Excitotoxicity

The neuronal depolarization associated with TBI results in excessive release of excitatory amino acids and glutamate is considered to be the principal contributor to excitotoxicity (Rothman and Olney, 1986). Glutamate can activate NMDA, AMPA and kainate receptors, leading to influx of sodium, calcium and hydrogen ions into the cell (Young, 1992). Swelling occurs due to osmotic shifts of water and the sharp sodium influx depolarizes the cell membrane, a process thought to be a major source for development of cytotoxic oedema (Young, 1992). NMDA-receptor antagonists have shown neuroprotective effects in experimental TBI (Faden et al., 1989) but clinical trials have failed to improve the outcome in patients (Morris et al., 1999).

Calcium

An intracellular increase of calcium is seen in CNS cells following TBI (McIntosh, 1993, Nilsson P. et al., 1993, Nilsson P. et al., 1996). The entry of calcium into the cell occurs principally through two pathways: via specific calcium channels (voltage-gated calcium channels and through the NMDA-channel following glutamate-mediated activation of the NMDA receptor and removal of magnesium ions) or through membrane disruption, e.g. via the membrane attack complex following activation of the complement cascade or by lipid peroxidation. The increased calcium influx leads to activation of several cellular pathways, including the calcium-dependent enzymes nitric oxide synthases and phospholipases, which cause production of free nitrogen radicals and ROS; this leads to further excitotoxic damage (McIntosh et al., 1998,Xiong et al., 2001). Strategies to limit secondary damage include blockade of calcium channels. The calcium-channel blocker Nimodipine has become standard treatment for patients with nontraumatic subarachnoid haemorrhage, but the results for clinical trials in TBI have been contradictory and pointing to a beneficial effect only in patients with traumatic subarachnoid haemorrhage (Harders et al., 1996, Langham et al., 2000, Teasdale et al., 1990). The effect of Nimodipine in experimental TBI has been investigated to a limited extent and only after clinical trials. The findings have been contradictory also in the experimental context (Ercan et al., 2001, Ustun et al., 2001) and the effects of Nimodipine in TBI are thus not clear.

Necrosis and Apoptosis

Both cellular necrosis and delayed apoptosis may affect CNS cells following brain injury (Ankarcrona et al., 1995, Raghupathi et al., 2000). Necrosis occurs in response to a toxic, or physical or ischemic insult, and is characterized by cellular swelling and membrane disruption in conjunction with lysis of nuclear chromatin. When groups of cells are affected simultaneously, large volumes of cellular contents are released within the damage tissues evoking chemical mediators and inflammatory responses within local areas. This perpetuation from necrotic tissue contributes to the demise of cells originally spared in the primary injury to the brain.

Apoptosis is a programmed cell death morphologically characterized by cell shrinkage, formation of an apoptotic nucleus, chromatin degradation and the formation of apoptotic bodies. The chromatin degradation can be enzymatically identified *in situ* using the TUNEL technique (Gavrieli et al., 1992). Apoptosis occurs in response to activation of cell signal pathways and also appears to contribute to the death of CNS cells following TBI (Clark et al., 1999, Raghupathi et al., 2000, Rink et al., 1995). It has been argued that the nature and/or intensity of the injury and the energy status of the cell regulate the mechanism of cell death resulting in apoptosis or necrosis (Raghupathi et al., 2000, Ankarcrona et al., 1995). Apoptosis is energy-dependent while the necrotic pathway for cell death is independent of energy. Morphological features of both necrosis and apoptosis may occur in the same neural cell, (Raghupathi et al., 2000, Rink et al., 1995) and it has been speculated that the determination for a cell to undergo necrosis or apoptosis is regulated by intracellular ATP levels; as long as ATP is present within the injured neuron, apoptotic pathways may be initiated, but once ATP is depleted (due to mitochondrial and/or PARP-activation) a shift toward necrotic pathways ensues (Raghupathi et al., 2000). PARP-inhibitors are drugs targeting the cellular suicide program, which have reduced brain damage following trauma in animal models (LaPlaca et al., 2001).

Inflammatory response

The mechanisms of secondary injury following TBI also include an inflammatory response. The immediate response of the brain to an insult is characterized by activation of macrophages/microglia in the brain parenchyma (Bellander et al., 1996, Holmin et al., 1995), accompanied by infiltration of activated leucocytes from the periphery (Soares et al., 1995). There are two pathways for leukocytes to enter the brain; through a disrupted blood brain barrier (BBB) caused by the primary insult, or via transendothelial migration and diapedesis. The inflammatory response in the brain following TBI is dependent on the increased release of proinflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 and an increased expression of adhesion molecules such as ICAM-1 (Carlos et al., 1997, Holmin et al., 1997), for a review se Arvin et al. (Arvin et al., 1996). For an experimental cerebral contusion, the inflammatory response peaks at day 4-7 after TBI (Holmin et al., 1995), a time which is associated with the later phase of the biphasic vasogenic oedema (Holmin and Mathiesen, 1995) and the delayed neurological deterioration, enlargement of the lesion and increased ICP frequently seen following cerebral contusions (Bullock et al., 1989, Cooper et al., 1979, Statham et al., 1989).

The inflammatory response following TBI is a potential target for therapeutic strategies. The formation of delayed vasogenic oedema is partly caused by the inflammatory reaction; it was decreased by use of the anti-inflammatory drugs Dexamethasone or Colchicine following experimental TBI (Holmin and Mathiesen, 1996, Hortobagyi et al., 2000). ICAM-1 antagonists and the immunosuppressive drug Cyclosporine are neuroprotective in experimental TBI (Baskaya et al., 2000, Alessandri et al., 2002). Post-traumatic treatment with Dexamethasone has in some experimental studies indicated a neuroprotective potential but phase III clinical studies have failed to confirm this effect (Alderson and Roberts, 2000, Narayan et al., 2002).. Colchicine, another drug with anti-inflammatory properties (Malawista, 1968; Molad, 2002), has been used for treatment of arthritis but has also been implicated in the context of experimental brain injury (Giulian et al., 1993, Giulian and Robertson, 1990, Holmin and Mathiesen, 1996). The use of anti-inflammatory drugs for TBI in patients remains to be further examined in clinical trials, especially with regard to the timing of anti-inflammatory treatment.

The complement system is activated both following experimental TBI (Bellander et al., 1996) and also in human ventricular cerebrospinal fluid (CSF) and brain tissue following head injury (Bellander et al., 2001, Kossmann et al., 1997). Activation of the complement system leads to increased vascular permeability, cytokine production and facilitation of phagocytosis (van Beek et al., 2003). Therapeutic strategies targeting the activated complement system following TBI are in the experimental stage and remain to be further examined in clinical trials.

Reactive Oxygen Species

Reactive oxygen species (ROS) are highly reactive molecules implicated in the pathology of traumatic brain injury through a mechanism known as oxidative stress (Demopoulos et al., 1980). ROS, such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$, OH^-), cause wide spread oxidative damage due to their ability to induce pathological changes at several molecular and cellular targets, including lipids, proteins, DNA, membrane structures and mitochondria (Evans, 1993, Hall, 1989, Hillered and Ernster, 1983, Lewen et al., 2000, Lewen and Hillered, 1998, Siesjo et al., 1989). The sources for free radical productions following TBI are several, including activated microglia, infiltrating inflammatory cells (rich of xantine oxidase that generates O_2^-), dysfunctional mitochondria and increased free iron due to

breakdown of extravasated haemoglobin (Halliwell and Gutteridge, 1986, Matsuo et al., 1995). The presence of free iron ions accelerates the formation of the most toxic ROS, the hydroxyl radical ($OH^{\cdot}$, OH^{-}), from superoxide and hydrogen peroxide, through the Fenton and Haber-Weiss reactions (Halliwell and Gutteridge, 1992, Sutton, 1985), which worsens the oxidative damage. The ROS-induced lipid peroxidation of the cell membranes results in membrane disintegration and increased microvascular permeability (Mathew et al., 1996).

Aminosteroids, e.g. Lazaroids, inhibit lipid membrane peroxidation by acting through free radical scavenging and membrane stabilization and thereby act as neuroprotectors after CNS injury (Kavanagh and Kam, 2001). The lazaroid Tirilazad Mesylate is reported to be beneficial in experimental trauma to the closed skull (Dimlich et al., 1990, Hall et al., 1988), and also in a focal cortical contusion model (Mathew et al., 1996). However, several phase III clinical trials have failed to show a beneficial effect in patients (Marshall and Marshall, 1995, Marshall et al., 1998). Steroids reduce free radical production and have commonly been used for treatment of patients with TBI although no clear benefit has been demonstrated for steroids in clinical trials except for patients with moderate focal contusions (Grumme et al., 1995, Narayan et al., 2002). Nitrone spin trap agents, such as PBN, S-PBN and NXY-059, form stabile adducts with carbon and oxygen centred radicals (Carney and Floyd, 1991), and have been shown to exert robust neuroprotection in experimental cerebral ischemia, and recently also in experimental TBI (Kuroda et al., 1999, Lewen et al., 2001, Marklund et al., 2001b). NXY-059 is now on clinical trials for stroke patients.

NITRIC OXIDE

Because of an unpaired electron, nitric oxide (NO) is a free radical, and its effects are largely mediated through other molecules that accept or share this odd electron. Target biological molecules include oxygen, other free radicals (i.e. superoxide), thiol groups and metals (Gonzalez-Zulueta et al., 2000). NO is involved in numerous regulatory functions, maintaining physiological homeostasis (Wink and Mitchell, 1998), but has also been found to be involved in a number of different pathological conditions, where its effect can be either protective or detrimental (Wink and Mitchell, 1998). Some biological effects of NO are illustrated in Fig. 3.

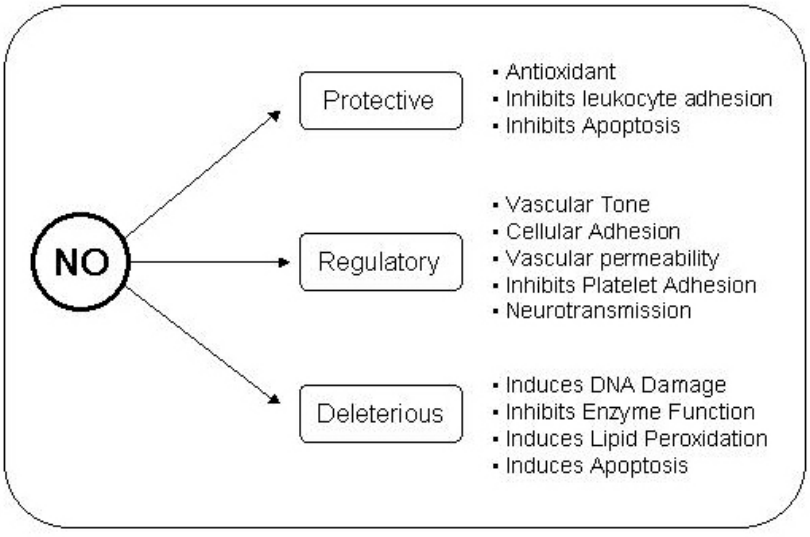

Figure 3. *Multifaceted biological effects of NO*

NO in itself is not a powerful cytotoxic agent; however it can render cells susceptible to other cytotoxic agents such as heavy metals, alkylating agents, and radiation (Wink and Mitchell, 1998). The labyrinth of divergent behaviours exhibited by NO has lead to confusion, and it has been suggested that the biological response of NO is dependent on where, when and how much NO is present or is being produced (Wink and Mitchell, 1998).

NO is synthesized by nitric oxide synthase (NOS), which catalyzes the five-electron oxidation of L-Arginine to L-Citrulline and NO (Marletta, 1993) (Fig. 4). Calmodulin, tetrahydrobiopterin (BH_4), and flavin-adenine dinucleotide (FAD) are three important co-factors involved in NOS-activity (Alderton et al. 2001).

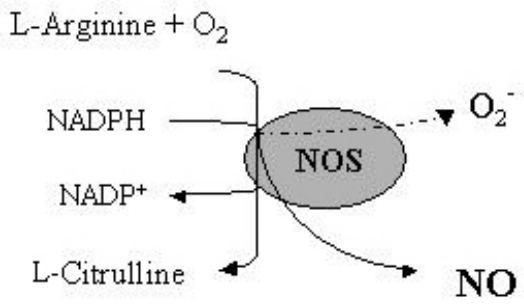

Figure 4. *A schematic illustration of the reaction of L-Arginine and oxygen to L-Citrulline and NO, catalyzed by nitric oxide synthase (NOS). Under L-Arginine depletion, NOS may also generate superoxide anion (O_2^-).*

Three different NOS isoenzymes have been described; endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (Bredt et al., 1990, Mayer et al., 1989, Yui et al., 1991). eNOS and nNOS are constitutively expressed in a calcium-calmodulin-dependent manner and have physiological roles in the regulation of cerebral blood flow (CBF), platelet aggregation, leukocyte adhesion and neuronal signalling (Garthwaite et al., 1988, Kubes et al., 1991, Ma et al., 1085, Murphy et al., 1993, Nozaki et al., 1993, Radomski et al., 1990), while iNOS is expressed only in pathological conditions and produces NO independent of intracellular calcium levels (Xie and Nathan, 1994). The biological effects of NO are divided into two major categories: direct and indirect. Direct effects are defined as those reactions fast enough to occur between NO and specific biological molecules. Indirect effects do not involve NO, but rather are mediated by reactive nitrogen oxide species (RNOS). RNOS is formed from the reaction of NO either with oxygen, to yield dinitrogen trioxide (N_2O_3) or with superoxide (O_2^-), to yield peroxynitrite ($ONOO^-$), N_2O_3 mediates predominantly nitrosative stress (S-Nitrosothiol), and peroxynitrite mediates oxidative stress (NT, lipid peroxidation, DNA damage, hydroxylation) (Wink and Mitchell, 1998). Direct effects occur at low concentrations of NO, whereas indirect NO-effects become significant at higher local NO concentrations (Radi et al. 2000, Wink and Mitchell, 1998). Cell types containing the constitutive NOS isoforms eNOS and nNOS, which produce NO dependent on the intracellular calcium level, generate low fluxes of NO for short periods of time; thus, direct effects of NO are the predominant chemistry and the indirect effects are limited (Wink and Mitchell, 1998). In the presence of iNOS, however, NO production is much greater and independent on calcium levels, and the indirect effects occurs (Wink and Mitchell, 1998). Cells or tissues close to an NO source may experience both indirect and direct effects of NO, while cells further away mainly will experience direct effects, since the NO concentration decreases as a result of diffusion and consumption.

NO may cause cytotoxicity by several mechanisms (Fig. 5). NO can damage DNA directly (Nguyen et al., 1992), inhibit mitochondrial respiration, (Bolanos et al., 1997, Dawson et al., 1992), or induce neuronal apoptosis via activation/ upregulation of pro-apoptotic mediators such as the transcription factor p53, Bax and caspases (Brune et al., 1998). NO reacts with superoxide to form the powerful neurotoxic oxidant, peroxynitrite, that causes protein tyrosine nitration, lipid peroxidation, DNA damage, mitochondrial dysfunction, and apoptosis (Hogg et al., 1993, Ischiropoulos et al., 1992a, Keller et al., 1998, Radi et al., 1994, Yamakura et al., 1998). DNA-damage leads to activation of DNA repairing enzyme, poly-ADP-ribose-polymerase (PARP), which consumes high amounts of energy and may facilitate collapse of energy metabolism and subsequent necrotic cell death (Salgo et al., 1995, Szabo and Ohshima, 1997). The formation of peroxynitrite is detrimental for the brain after TBI (Hall et al., 1999, Whalen et al., 1999).

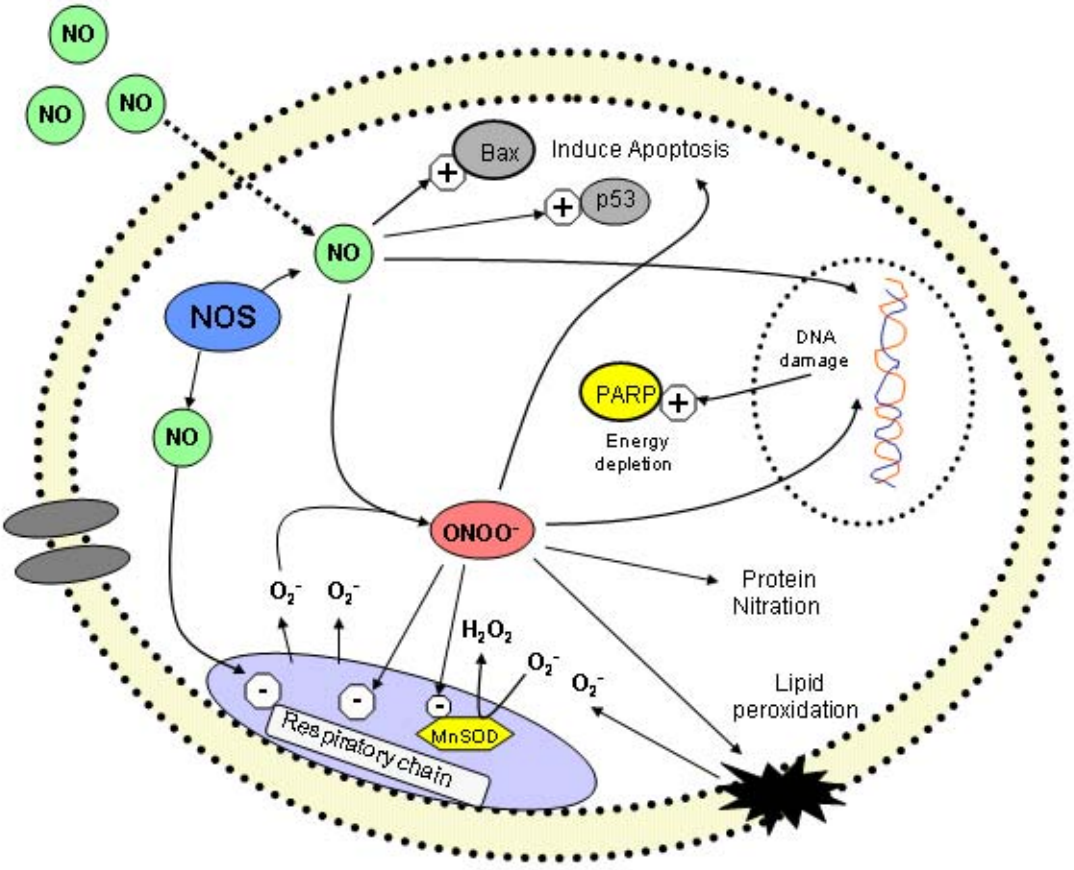

Figure 5. *Cytotoxic effects of NO. NO can directly, or via peroxynitrite ($ONOO^-$), induce DNA-damage, mitochondrial dysfunction (via inhibition of complexes in the respiratory chain), and neuronal apoptosis. DNA-damage leads to activation of DNA repairing enzyme, poly-ADP-ribose-polymerase (PARP), which consumes high amounts of energy, which facilitates collapse of energy metabolism, and subsequent necrotic cell death. Mitochondrial dysfunction leads to superoxide (O_2^-) production. Additionally, peroxynitrite causes protein tyrosine nitration, lipid peroxidation and inactivates superoxide consumption via inhibition of Mn Superoxide Dismutase (MnSOD), resulting in increased oxidative injury.*

The NOS isoenzymes produce NO. However, under L-arginine depletion, superoxide anion is concomitantly formed, promoting peroxynitrite formation, through the uncoupling of NADPH consumption from arginine oxidation (Fig. 4). This process has been shown to occur for all

three NOS isoenxymes (Vasquez-Vivar et al., 1998, Xia et al., 1996, Xia and Zweier, 1997, Xia et al., 1998). Thus, increase in NOS activity is a source of both nitrogen and oxygen radicals.

There are also several potentially protective intracellular effects of NO (Fig.6). The RNOS N_2O_3 mediates S-nitrosylation of thiol groups on the NMDA receptor's redox modulator site, which downregulates the NMDA receptor-activity, and thereby attenuating excitotoxicity (Lipton et al., 1993). In addition, NO may also inhibit apoptosis, via N_2O_3 -mediated S-nitrosylation of caspase-3 or by cGMP-mediated inhibition of apoptotic signalling (Estevez et al., 1998, Kim et al., 1997).

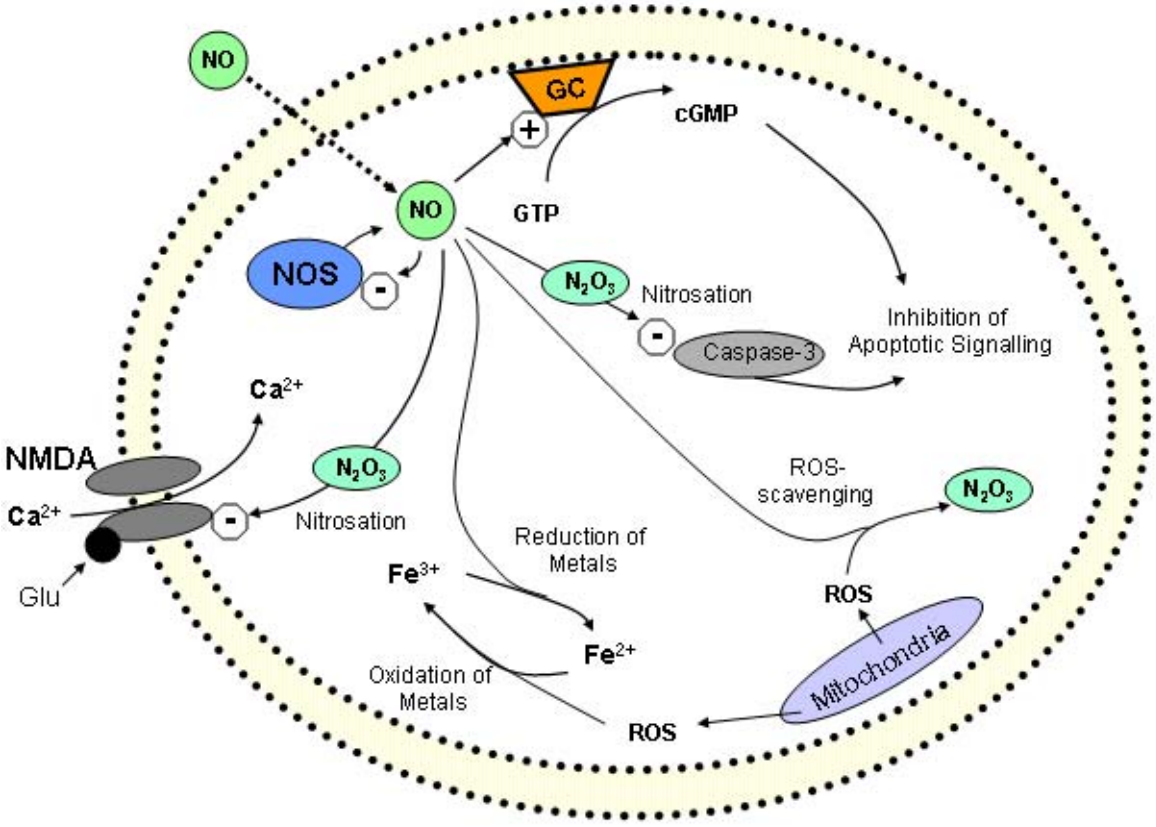

Figure 6. *Cytoprotective effects of NO. NO-mediated nitrosation of the NMDA receptor by N_2O_3 downregulates its activity and reduces Ca^{2+} influx and related toxicity. NO reacts with reactive oxygen species (ROS) and with metallo-species that have been oxidized by ROS, and thus protects from ROS induced damage. NO may also inhibit apoptotic pathways via cGMP (Guanylate Cyclase (GC)-activation) and via nitrosation of Caspase-3.*

Thus, the consequences of NO for the brain depend on where and when it is synthesized. Furthermore, the cellular sources, temporal profile and dependency of intracellular calcium levels differ for the three NOS isoenzymes and NO may therefore differently affect the pathophysiological reactions in the injured brain (Iadecola, 1997).

The induction of iNOS following TBI may also be influenced by the proinflammatory cytokines (TNF-α, IL-1β and IL-6) that are released after brain damage. TNF-α and IL-1β are two major inducers of iNOS mRNA transcription and act through activation of NFκB pathway (Ganster and Geller, 2000; Kleinert et al., 2000). IL-6 has also been implicated as an additional factor for induction of iNOS mRNA, acting through other signalling pathways (Ganster and Geller, 2000; Kleinert et al., 2000). Consequently, iNOS is a possible actor in inflammatory

reactions and may catalyze synthesis of NO in the injured brain (Iadecola, 1997, Minc-Golomb et al., 1994). Several drugs with neuroprotective potential may theoretically also affect the expression and upregulation of iNOS, which hypothetically may contribute to their mechanisms of neuroprotection. Neuroprotective and/or anti-inflammatory drugs such as Colchicine, Dexamethasone, Tirilazad Mesylate and Nimodipine have different putative mechanisms of action but their effects on iNOS synthesis in TBI are not clear.

The involvement of NO in the pathogenesis of neuronal damage in cerebral ischemia is complex (Iadecola et al., 1997). Several studies have demonstrated that NO derived from eNOS seems to be beneficial for the injured brain (Huang et al., 1996) whereas effects of nNOS- and iNOS-derived NO has been suggested to be mainly detrimental (Hara, 1996 #283, Huang et al., 1994, Iadecola et al., 1997, Iadecola et al., 1995b). There are numerous findings showing that the temporal pattern of NO-production is of importance for lesions induced by cerebral ischemia (Iadecola, 1997). It has been suggested that NO produced in the endothelial cells or in perivascular neurons immediate after the trauma is mainly protective due to vasodilatation and increased cerebral blood flow (CBF), decreased platelet activation, diminished leukocyte adhesion (Hlatky et al., 2003, Iadecola, 1997). It has also been demonstrated that NO-donors are protective during reperfusion, by quenching ROS and protect against ROS-mediated reperfusion injury, (Mason et al., 2000). Later in the post traumatic phase, however, NO derived from parenchymal neurons or from inflammatory cells seems to be mainly detrimental (Iadecola, 1997). NO can also induce brain injury via effects on astrocytic function, since NO is capable of attenuating astrocytic glutamate uptake (Ye and Sontheimer, 1996). In global ischemia, the induction of iNOS occurs predominantly in reactive astrocytes, resulting in the release of NO, which can increase perineuronal glutamate levels. The resulting long-term potentiation of NMDA receptors is detrimental in itself but may also cause additional NO synthesis from calcium dependent nNOS in neurons and further cytotoxic effect.

NO is thought to be implicated in the pathophysiology of secondary damage also in TBI. Induction of NOS has been described in experimental TBI-models (Clark et al., 1996b, Sakamoto et al., 1997) and increased nitrite and nitrate concentrations in cerebrospinal fluid and plasma have been found after head injury in humans (Clark et al., 1996a). However, the dynamics, compartmental distribution and pathophysiological potential of NOS in TBI are not clear and studies on the pathophysiological features of head injuries have produced conflicting data, supporting both a beneficial and a detrimental role for NOS induction (Mesenge et al., 1996, Sinz et al., 1999, Wada et al., 1998, Wada et al., 1999). An understanding of the cellular sources and relative compartments of the differently regulated NOS isoforms is required for the analysis of NO in the pathophysiology of TBI.

AIMS OF THE STUDY

To characterize and describe the dynamics and cellular sources of the three NOS isoforms in a model of experimental brain contusion.

To analyze the expression, possible upregulation and cellular sources of the NOS isoenzymes following brain contusions in humans.

To analyze the possible effects of posttraumatic NOS inhibition with and without a nitrone spin trap agent after an experimental brain contusion

To determine the effects of iNOS on secondary neuronal injury in experimentally induced brain contusion, using a selective iNOS-inhibitor.

To further investigate the neuroprotective capacity of Colchicine, Dexamethasone, Tirilazad Mesylate and Nimodipine by analysis of their effect on iNOS expression, cellular apoptosis, neuronal degeneration and survival, following an experimentally induced brain contusion.

MATERIALS AND METHODS

All experiments were approved by the local animal care and use committee (I, II, III and IV) or the local committee at the Karolinska Hospital (V)

ANIMALS (I AND III-V)

Adult Sprague-Dawley rats (B&K Universal Sollentuna, Sweden) were used. All animals were provided with food and water ad libitum before and throughout the experimental procedures.

PATIENTS (II)

The analysis of human specimen was performed in contused brain tissue specimens taken from eight consecutive patients who underwent surgical treatment for brain contusions.

TISSUE PREPARATIONS

Experimental contusions (I and III-V)

A stereotactic weight-drop cortical contusion model, originally described by Feeney *et al.* (Feeney et al., 1981), was modified and used for induction of cerebral contusion (Fig. 7). Rats weighing 190-360 grams were anesthetized by injecting 0.2 ml of Hypnorm™ (0.315 mg /ml fentanyl citrate and 10 mg/ml fluanisone) (I and III-V) and 0.2 ml Midazolam™ (1 mg/ml midazolam) (III and IV) intramuscularly. In addition, 0.05 ml of Xylocain™ (5 mg/ml lidocaine) was injected subcutaneously in the sagittal midline of the skull before the skin incision was made. The rats were placed in a stereotactic frame and a craniotomy (2 mm in diameter) was drilled under microscopic guidance at a point 2 mm posterior and 2 mm lateral, right (I, IV and V) or left (III) to the bregma. Aseptic techniques were used throughout the surgical experiments. A standardized small parietal contusion was performed by letting a steel rod, weighing 24 grams, fall onto a piston with a diameter of 1.8 mm and in contact with the intact dura mater, from a height of 6.5 cm (Fig. 7). The maximal compression of the brain tissue was 3 mm. In sham-operated animals, a craniotomy without contusion was performed. The animals showed no deficits in motor or balance function after the trauma and presented normal grooming and feeding behaviour after the postoperative and anesthetized period.

In paper III-V, animals received one or a combination of two drugs (III), or control substance, post trauma. The route of administration, dose, interval, and duration of drugs is presented in Table 1. The animals were sacrificed at various times from 6 hours at earliest and 14 days at latest after trauma. For histological analysis, the brain was dissected, frozen in isopentane containing dry ice, and stored at -70°C for later analysis. Coronal cryosections (14μm) were cut through the lesion, thawed onto object glasses and stored at -20°C. Slides for quantifications were cut exactly through and in the equivalent localizations of the traumatized area.

For NOS activity measurements (III and IV), the animals were sacrificed at 24 hours post trauma, the brain were quickly dissected and frozen in cold isopentane. The contused area was easily recognized and well defined in all rats. The contused area and adjacent grossly normal cortex was dissected out and stored at -20°C for later analysis (see below). An equally large

piece from the same anatomical area was dissected from normal brain to serve as control. In paper IV, an equally large piece from the same anatomical area was dissected also from the contralateral hemisphere.

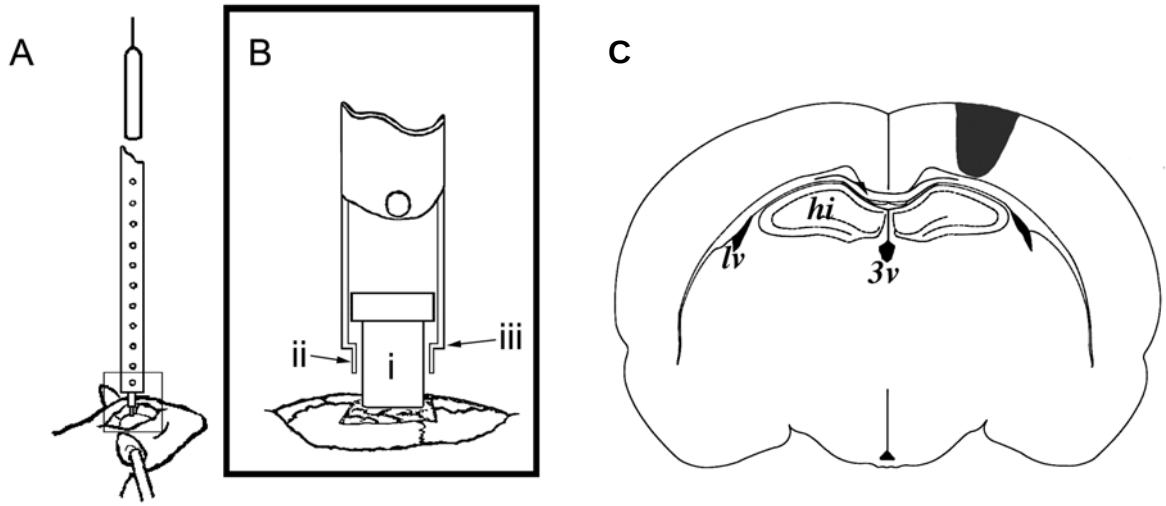

Figure 7. *Schematic illustration of the contusion device showing the weight suspended in the guidance tube (transparent for clarity), positioned over the exposed skull of a rodent (A). The marked area in A is shown magnified (transparent for clarity) in B. The piston (i) is pushed up in the sleeve (ii) by the exposed dura mater and the force of the falling weight is subsequently transferred to the neocortex by the piston. Finally, the cuff (iii) of the sleeve stops the downward movement of the piston. C is showing a schematic illustration of the lesion (black) in a coronal section 2 mm posterior to bregma. Hippocampus (hi), lateral ventricle (lv), 3rd ventricle (3v).*

Human contusions (II)

Contused brain tissue was obtained from patients who were surgically treated for brain contusions with an interval of 3 hours to 5 days after trauma. The biopsies were snap-frozen in liquid nitrogen and stored at -70°C. Care was taken to obtain representative tissue from injured but non-hemorrhagic, cortical areas in the frontal or temporal lobes.

Table 1. *Drugs used in the present study*

Drug	Dose	Set off	Dose interval	End	Source	Reference
L-NAME	100 mM, 0.25µl/h	15 min p.t.	Infusion	X [a]	Sigma	(Packer et al., 2003)
L-NIL	5 mg/kg	15 min p.t.	12 hours	12 h p.t	Sigma	(Zhang J. Y. et al., 1229)
S-PBN	100 mg/kg	15 min p.t.	2 hours	4 h p.t	Sigma	(Yang et al., 2000)
Colchicine	0.2 mg/kg	4 hours p.t.	24 hours	X [a]	Apo	(Holmin et al, 1996)
Dexamethasone	1 mg/kg	4 hours p.t.	24 hours	X [a]	Apo	(Holmin et al, 1996)
Tirilazad Mesylate	10 mg/kg	4 hours p.t.	24 hours	X [a]	Upjohn	(Sanada et al., 1993)
Nimodipine	360 µg/kg	4 hours p.t.	12 hours	X [a]	Bayer	(LINFO, 2005)

X [a] – animals received the drug during the entire experiment (until euthanasia). Apo (Apoteksbolaget AB, Sweden), Bayer (Bayer AB, Germany), Sigma (Sigma-Aldrich, Germany), Upjohn (Upjohn Lab., USA) p.t.-post trauma

IMMUNOHISTOCHEMISTRY (I-V)

Single labelling

All primary and secondary antibodies were diluted in 1% and 4% bovine serum albumin (BSA), respectively. The indirect or double indirect peroxidase method was used for all single labelling except for NeuN (neuronal nuclei) staining, where an avidin-biotinylated enzyme complex (ABC) peroxidase kit was used (see below). In brief, the sections were air-dried in RT for 1.5 hours and fixed in 4% formaldehyde or acetone, washed and incubated in 0.3 % hydrogen peroxide to quench endogenous peroxidase. The sections were then incubated over night at +4°C with the primary antibody (Tables 2 and 3). The next day, the sections were washed and peroxidase conjugated secondary- and sometimes tertiary- antibodies were applied for 1 hour each, with washing in between. Normal goat serum (NGS), normal rabbit serum (NRS) or 1% bovine serum albumin (BSA) was used to prevent non-specific conjugate binding. When staining rat tissue, the conjugates were preabsorbed over night at +4°C with normal rat serum to minimize cross reactivity with rat immunoglobulin (Ig). After washing, the bound peroxidase was visualized by incubation for 5 minutes with a diaminobezidine (DAB) substrate kit (brown) (SK-4100, Vector Laboratories Inc., CA, USA). Sections were counterstained with Meyer's haematoxylin (blue), dehydrated and mounted with DPX (Distrene 80, dibutyl phthalate, xylene, BDH Laboratory Supplies Poole, UK).

For NeuN staining, a Vectastain Elite ABC peroxidase kit (Vector Laboratories) was used. In brief, before the sections were incubated with the polyclonal NeuN primary antibody (1/500, Chemicon International, Ternecula, CA.), blocking serum (PBS plus 0.3 % Triton plus 1% BSA) was used to prevent non-specific conjugate binding to the primary antibodies. An avidin-biotin blocking step was then performed with ABC, to prevent non-specific conjugate binding to endogenous biotinylated proteins. The sections were then incubated with the primary antibody NeuN over night at +4°C. The indirect peroxidase method was used for detection of the primary antibodies. Biotin-conjugated goat anti-rabbit immunoglobulin (dilution 1:200; Jackson ImmunoResearch Laboratories) was used as conjugate. The sections were incubated with the ABC standard kit. The bound peroxidase was detected via incubation with a DAB substrate kit. Finally, the sections were dehydrated and mounted with DPX.

Nitrotyrosine (NT) is a indirect relatively specific biomarker for peroxynitrite-induced cellular damage (Beckman et al., 1994), as nitration of the ortho position of tyrosine is a major product of peroxynitrite attack on proteins (Ischiropoulos et al., 1992b). Recent studies have indicated that the pathways for formation of NT are multiple. NT may, except for peroxynitrite, also be generated e.g. by autooxidation of NO (N_2O_3), heme, or hemoprotein catalyzed hydrogen peroxide-dependent oxidation of nitrite (reviewed in Tarpey et al., 2004). Thus, the presence of NT is not a specific "footprint" for peroxynitrite formation in vivo, but a result of both nitrosative and oxidative cellular stress (Tarpey et al., 2004), which should be considered in the analysis of drug-related effects of NT formation. However, peroxynitrite is a major source of NT formation, and significant differences in peroxynitrite formation is suggested to be reflected in the level of NT formation (Ischiropoulos et al., 1992b) . NT formation was assessed at 24 hours after the trauma using immunohistochemistry with NT-specific antibodies. Sections were fixed in 4 % formaldehyde and the staining procedure was performed according to the immunohistochemistry protocol from the company, using the indirect peroxidase method with DAB substrate. Positive control was chosen according to the company recommendations; preincubation with 1 mM sodium nitrite, 1 mM hydrogenperoxide in 100 mM sodium acetate,

pH 5.0. BSA instead of primary antibody (NT) was used for negative controls. The sections were counterstained with Meyer's haematoxylin, dehydrated and mounted with DPX.

Double labelling

In paper II, immunohistochemical peroxidase double labelling was used. In brief, the same procedure as for single labelling with ABC peroxidase kit was performed except for that the bound peroxidase for the first primary antibody was visualized with DAB-*nickel* substrate kit (black) (SK-4100; Vector laboratories). Before mounting, the staining procedure was repeated for incubation with the second primary antibody, followed by the indirect peroxidase staining method with ABC. The bound peroxidase was detected via incubation for 3 minutes with a Vector NovaRED substrate kit (red) (SK-4800; Vector Lab). Finally, sections were counterstained with haematoxylin and mounted with DPX.

Table 2. *Primary antibodies to rat antigens used in the present study*

Antibody	Specificity	Ig class, host	Dilution	Source	Reference
iNOS	iNOS	IgG-fr., rabbit	1:800	Transduction	(Lyons et al., 1992)
nNOS	nNOS	IgG-fr, rb/mo	1:800	Transduction	(Bredt et al., 1991)
eNOS	eNOS	IgG_1, mouse	1:800	Transduction	(Chen P. F. et al., 1994)
W3/13	Neutrophils, pan T cells	IgG_1, mouse	1:1000	Sera-Lab	(Williams et al., 1977)
ED-1	Monocytes/macrophages	IgG_1, mouse	1:4000	Serotec	(Lassmann et al., 1993)
NeuN	Neurons	IgG_1, mouse	1:500	Chemicon	(Suhonen et al., 1996)
GFAP	Astrocytes	IgG_1, mouse	1:500	a	(Jie et al., 1986)
Nitrotyrosine	Nitrotyrosine	IgG-fr., rabbit	1:250	Upstate	(Ischiropoulos 1992b)
Albumin	Albumin	IgG-fr., rabbit	1:1000	Cappel	

IgG-fr - IgG-fraction, rb/mo – rabbit or mouse

a – Kindly provided by Prof. V. Peter Collins, Dept. of Pathology, Karolinska University Hospital, Stockholm, Sweden

Transduction (Transduction Laboratories, Lexington, KY, USA), Sera-Lab (Sussex, UK), Serotec (Oxford, UK), Chemicon (Chemicon International, Temecula, CA, USA), Upstate (Upstate Biotechnology, Lake Placid, NY, USA), Cappel (Cappel Research Products, NC, USA)

Table 3. *Primary antibodies to human antigens used in the present study*

Antibody	Specificity	Ig class, host	Dilution	Source	Reference
iNOS	iNOS	IgG-fr., rabbit	1:800	Transduction	(Lyons et al., 1992)
nNOS	nNOS	IgG-fr., rabbit	1:800	Transduction	(Bredt et al., 1991)
eNOS	eNOS	IgG_1, mouse	1:800	Transduction	(Chen P. F. et al., 1994)
GFAP	Astrocytes	IgG_1, mouse	1:500	Dakopatts	
NeuN	Neurons	IgG_1, mouse	1:500	Chemicon	(Suhonen et al., 1996)
N-Elastase	Neutrophils	IgG_1, mouse	1:50	Dakopatts	
ED-1	Monocytes/macrophages	IgG_1, mouse	1:4000	Serotec	(Lassmann et al., 1993)
Transferrin	Oligodendrocytes	IgG-fr, rabbit	1:1000	Nordic	

IgG-fr.- IgG-fraction

Dakopatts (Glostrup, Denmark), Nordic Immunological Laboratories (Tilburg, The Netherlands), Transduction (Transduction Laboratories, Lexington, KY, USA), Serotec (Oxford, UK), Chemicon (Chemicon International, Temecula, CA, USA)

FLUORO-JADE HISTOCHEMISTRY (III-V)

Fluoro-Jade (FJ) is a fluorescent dye that specifically labels and histochemically localizes degenerating neurons (Schmued et al., 1997). Staining for Fluoro-Jade was performed to detect neuronal degeneration in the experimental groups after trauma. Prior to staining, the sections were dried for 1 hour and thereafter fixed in 4% buffered proformaldehyde for 10 minutes at room temperature. After washing in PBS, the sections were rinsed in distilled water and incubated with 0.00002% Fluoro-Jade in 0.1% acetic acid for 30 minutes in room temperature in coplin jar on shaker. After washing in distilled water, sections were dried on hot plate (approximately 50˚C) and mounted with DPX.

TUNEL HISTOCHEMISTRY (III-V)

Identification of programmed cell death in situ was analyzed via specific labelling of nuclear DNA fragmentation (TUNEL) (Gavrieli et al., 1992) The sections were prepared in the same way as described for immunohistochemistry except that they were post-fixed in ethanol: acetic acid 2:1 for 5 minutes at -20˚C prior to the staining procedure. The TUNEL reaction was carried out with the "In situ cell death detection kit, fluorescein" (Boehringer Mannheim, Bromma, Sweden) at 37˚C for 30 minutes. The slides were washed in PBS and mounted with Glycerol: PBS (2:1) and stored at + 4˚C. For fluorescence double labelling of TUNEL and cellular markers, the same TUNEL procedure was followed, but before mounting, sections were incubated with NGS for 30 minutes and the primary antibody was applied and incubated over night at + 4˚C. After washing, an indocarbocyanine (Cy3)-conjugated Fab_2 fragments with appropriate species reactivity's was applied for 1 hour at RT. The sections were thereafter washed and mounted with Glycerol: PBS (1:1).

EVALUATION OF IMMUNO-, FJ- AND TUNEL HISTOCHEMISTRY

Experimental contusion

Immunoperoxidase stainings (I and III-V) were evaluated by light microscopy (Leica DM400B^{Tm}). Quantification was performed by manual counting of cells, in a defined anatomical area (Fig 8), under high power (400-1000×) or by computerized image analysis (III-V), using KODAK 1D Image Analysis Software (Eastman Kodak Company, Scientific Imaging Systems Rochester, NY 14650). Photomicrographs of the perilesional cortex (penumbra) (Fig. 8) and the contralateral hemisphere were taken by a Leica DFC 320 camera in low power (50×), and saved in a software program (Adobe Photoshop 7.0). In paper I, a rectangular area of 5.46 mm^2, which extended 2.6 mm laterally from the fissure longitudinalis cerebri and 2.1 mm inferiorly from the brain surface, was evaluated, as a replacement for the analyzed area in paper III-V. A semi-quantitative analysis and evaluation of nitrotyrosine (NT) stainings (III and IV) were performed only within the contusion and the penumbral zone. The level of NT staining was thereafter scored as described by Hooper et al 2000 (Hooper et al., 2000); 0 = none; 1 = <10 positive discrete loci of staining in a brain coronal cross section; 2 = >10<50 scattered discrete loci or areas of weak staining; 3 = extensive areas of strong staining.

FJ- and TUNEL stainings (III-V) were evaluated by fluorescence microscopy in a Leica DMRB^{Tm} fluorescence microscope. Quantification was performed by manual counting of cells, in a defined anatomical area (Fig. 2), under high power (400-1000×). TUNEL –positive cells were differentiated as being apoptotic or necrotic based on TUNEL-positive staining together with specific histological determination of two or more of the classic morphologic hallmarks of apoptosis; membrane blebbing, chromatin condensation, nuclear shrinkage and cytoplasm

condensation and disintegration. Consequently, apoptotic cells exhibited intense nuclear staining and breakdown of the cell surface into spherical apoptotic bodies. Swollen, diffusely TUNEL-stained cells not exhibiting apoptotic morphology were considered to be necrotic. Only TUNEL-positive cells with apoptotic morphology were counted and will in the current thesis be referred to as TUNEL-positive cells.

Evaluations of fluorescence double labelling (I and III-V) were performed by fluorescence microscopy, using two different Leica filter cube L4 (excitation filter: 450-490 nm, suppression filter: 515-560 nm) to distinguish between Cy3- and FITC-labelled cells. To determine the coexpression ratio of the two antigens, a certain number of cells were analyzed by alternating the filter cubes for each cell.

To study the presence of vasogenic oedema in this model, horizontal sections through the contusion were stained for albumin. Evaluation of albumin staining was performed in light microscopy (Leica DM400B^{Tm}) and under high-power (200-400×). Absence of albumin extravasation was defined as distinct intraluminal albumin immunoreactivity. Presence of albumin extravasation was defined as intense, diffuse parenchymal albumin immunoreactivity with non-discernible intraluminal staining.

All slides were blinded for quantification and semi-quantitative analysis in the evaluation of immuno- FJ- and TUNEL histochemistry.

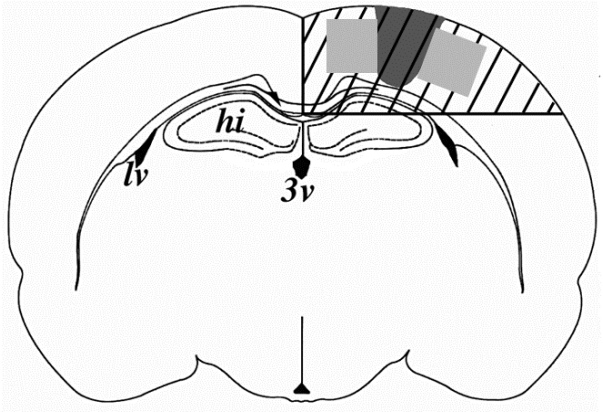

Figure 8. *A schematic coronal section through the brain illustrating the site of the contusion (black), the analyzed area for Fluoro-Jade, TUNEL, iNOS and nitrotyrosine stainings (striped), and the areas analyzed for NeuN positive cells in the perilesional cortex (light grey). Hippocampus (hi), lateral ventricle (lv) and 3rd ventricle (3v).*

Human contusions

In the human paper (II), immunoperoxidase stained cells were evaluated by light microscopy (Leica DM400B^{Tm}). Quantification was performed by manual counting of cells, in non-necrotic areas, in a total of nine high-power (400x) fields per section, randomly chosen from three selected areas in low power magnification (50×). Morphological characterization of cells were used to count iNOS, nNOS and eNOS-positive cells was as follows; Monocytes were defined as large round/oval cells with round/oval nuclei and abundant cytoplasm. Neurons were defined as large round/oval cells with cantered, large, round nuclei. Endothelial cells were defined as small, lined cells with round /oval nuclei and abundant cytoplasm. Polymorphonuclear cells (PMN) were defined as round/irregular cells with segmented or U-shaped nuclei and abundant cytoplasm. The morphological criteria were confirmed via analysis of sections that were double stained for cellular markers and iNOS or nNOS.

For evaluation of immunoperoxidase double labelling assays, light microscopy was used. Cells that were positive for iNOS or nNOS were incubated with DAB-nickel and demonstrated a grey/black-staining pattern. Cells that were positive for the actual cellular marker thereby were incubated with Vector NovaRED and demonstrated an orange/red staining pattern. Cells that reacted with the two primary-antibodies demonstrated a mixed staining pattern, with

grey/black and orange/red in the cytoplasm or nucleus. The relative proportion (%) of different cellular sources of iNOS was estimated by counting the number of cells that expressed the antigens for the two primary antibodies (iNOS and a cell-specific marker) from a total of 50 iNOS cells in each section.
All slides were blinded for quantification measurements.

NOS ACTIVITY MEASUREMENTS (III AND V)

NOS activity was measured at 24 hours post trauma by the conversion of L- [U-^{14}C] arginine to L- [U-^{14}C] citrulline as described (Salter et al., 1991). This method has been commonly used for NOS activity measurements. Recent findings have demonstrated the importance of accurate concentration of calcium chelator when assessing the calcium independent NOS enzymatic activity, to make sure that calcium-free conditions are achieved (Louin et al., 2004). Accordingly, we used the higher concentration of EGTA (2 mM) for all assessments in the present study. Briefly, the frozen tissue was homogenized in 2 volumes of ice-cold buffer containing 320 mM sucrose, 10 mM Hepes, 0.1 mM EGTA, 1 mM DL-dithiothreitol, 10 µg/ml trypsin inhibitor, 10 µg /ml leupeptin, 100 µg/ml phenylmethylsulfonyl fluoride and 2 µg/ml aprotinin (adjusted to pH 7.2 at 20C with 1M HCl). The homogenate was centrifuged at 10,000 g for 30 minutes at 4°C and the supernatants were collected and stored on ice before analysis. To measure NOS activity in the supernatants, tubes filled with 100 µl of a buffer, consisting of 50 mM potassium phosphate (pH 7.2), 50 mM L-valine, 100 micro M NADPH, 1 mM L-citrulline, 20 am L-arginine and L- [U-^{14}C] arginine (150,000 dpm) and 1.2 mM $CaCl_2$, were pre warmed to 37°C. 20 µl of the supernatants was added to the tubes. Duplicate incubations of each sample were done for 10 minutes at 37°C in the presence or absence of EGTA (2 mM) or EGTA plus N^{ω}-monomethyl-L-arginine (2 mM each) to determine the levels of the calcium-independent and the calcium-dependent activities. The reaction was terminated by removal of the substrate and dilution with 1.5 ml of 1:1 (v/v) water/Dowex AF 50W-X8 (pH 7.5). 5 ml of water was added to the incubation mix and 2 ml of the supernatant was removed and examined for presence of L- [U-^{14}C] citrulline by liquid scintillation counting. The level of citrulline was estimated and expressed as pmol per gram of tissue (wet weight) per minute.

PHARMACOLOGICAL CONSIDERATIONS

The ability for a drug to penetrate the blood brain barrier (BBB) has been described as an important feature for successful pharmacological neuroprotection (Doppenberg et al., 2004). Drug administration directly to the CSF circumvents this requirement, but is not commonly preferred over intravenous administration, since more side effects and risks have to be considered for this route. A number of substances have shown neuroprotection in experimental studies, despite a poor ability to penetrate the BBB (Hall, 1995, Kuroda et al., 1999, Marklund et al., 2001a, Marklund et al., 2001b). This might be explained by a drug action restricted to the blood-endothelial interface, or active metabolites may penetrate the BBB, or the drug may enter the brain through a disrupted BBB. BBB breakdown is observed and a pathological event almost instantly after TBI and may persist for up to 24-72 hours (Baldwin et al., 1996, Tanno et al., 1992). The initial breakdown may except for mechanical disruption induced by the trauma be caused by a disruption of tight junctions in arterioles and arteries, the later may be caused by the immediate increase of arterial blood pressure that follows the impact (Golding, 2002).

We assumed that the drugs used in the present study, except actions at the blood-endothelial interface, also had a possibility to enter the brain parenchyma through the disrupted BBB.

Drug administration

Subcutaneous (s.c.)-, intramuscular (i.m.)-, intraperitoneal (i.p.) injections (III-V) and intraventricular infusions (i.t.) (III) were used to deliver drugs to the animals. S.c. injections were used to administrate local anaesthetic substance (Xylocain™). I.m. injections were used to administrate central anaesthetic substances (Hypnorm™ and Midazolam™).

I.p. injections were used to administer therapeutic drugs (2-sulfo-phenyl-N-*tert*-butyl nitrone (S-PBN), L-*N*-iminoethyl-lysine (L-NIL), Dexamethasone, Colchicine, Tirilazad Mesylate and Nimodipine. In general, the drugs were administered once or twice daily and for a prolonged period, and i.p. injection was therefore chosen over intravenous (i.v.) administration. The peritoneal cavity offers a large absorbing surface, and water-soluble drugs injected i.p. are often absorbed rapidly but about four times slower than after an i.v. injection (Waynforth and Flecknell, 1992). The injection was made into the lower left quadrant of the abdomen, to avoid injury to vital organs. The needle was inserted vertically, only the tip penetrated into the peritoneal cavity. Aspiration into the syringe was carried out before the injection to detect possible puncture of the intestine and subsequent error in drug delivery. All i.p. injections were carried out by one person (C.G), to limit individual variations. I.p. injection of NaCl (9mg/ml, 1.5 ml) was used as control substance for all drugs except for L-NAME, where PBS was used.

Miniosmotic pumps (Azlet model 2004) were used to infuse N^G -nitro-L-arginine methyl ester (L-NAME) (diluted in sterile PBS) in order to give a constant inhibition of the NOS isoenzymes. L-NAME is reported to be biologically stabile for 16 days (Packer et al., 2003). L-NAME was administrated into the lateral ventricle, since the drug penetrates well into the brain parenchyma following intraventricular administration (Greenberg et al., 1997). A small craniotomy (0.5 mm in diameter) was drilled under microscopic guidance and the tip of the infusion kit was placed into the right lateral ventricle according to the following stereotactical coordinates; 0.9 mm posterior, 1.4 mm lateral to bregma, and 3.6 mm below dura mater.

Dose and time of drug administration

In general, all drugs were administrated post trauma in order to mimic the clinical conditions for TBI patients. The doses were optimized from previous experimental studies where the drugs have shown neuroprotective properties (see references in Table 1). The sources, doses and routes of administration are presented in Table 1.

NOS-INHIBITION

There is a multiple range of NOS inhibitors described in the literature and in use as pharmacological tools (Southan and Szabo, 1996). Inhibition of NOS isoforms may be achieved in several ways; Targeting the differential co-factor requirement (Calmodulin, BH4, FAD), the substrate requirements (L-Arginine uptake blockers or Arginase), or pharmacological agents that are selectively taken up by cells expressing various isoforms of NOS (7-nitroindazole) or NOS-inhibitors targeting the active site of the NOS with isoform specificity (Southan and Szabo, 1996). Inhibitors targeting the active site for NOS may be based on L-Arginine, other amino acids (L-citrulline and L-Lysine), or non-amino based NOS-inhibitors (e.g. guanidine, 7-

nitroindazoles) (Southan and Szabo, 1996). NOS-inhibitors that have been widely used in experimental cerebral ischemia and also some TBI-studies are the L-Arginine-based inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), the guanidine derivate Aminoguanidine (AG) and the indazole 7-nitroindazole (7-NI) (Iadecola, 1997, Wada et al., 1998, Wada et al., 1999). Two other NOS inhibitors, with a higher selectivity for iNOS over eNOS and nNOS; 1400W and N^6-iminoethyl-L-lysine (L-NIL) have been previously used in pharmacological treatment of experimental TBI (Jafarian-Tehrani et al., 2005, Sinz et al., 1999).

There has been differences in the literature on NOS inhibitors of what constitutes selectivity (10-fold or 100-fold) and how selectivity is defined (ratio of IC_{50} values at constant substrate concentration or ratio of K_i values or ratio of pharmacologically-effective doses) or determined effects on isolated enzymes or in cells/tissues or *in vivo* pharmacological properties (Alderton et al., 2001). In a review by Alderton et al 2001 (Alderton et al., 2001), the level of selectivity is defined on the basis of relating their potency under identical conditions in the physiological range (L-Arginine concentration etc) The inhibitor was defined as *non-selective* if the selectivity is less than 10-fold, *partially selective* if the selectivity is 10–50-fold, and *highly selective* if the selectivity of the compound is over 50-fold (Alderton et al., 2001). According to Alderton et al.´s definition, the selectivity for some NOS-inhibitors would be as presented in Table 4.

Table 4. *The selectivity towards the NOS isoforms of some NOS inhibitors*

NOS-inhibitor	iNOS vs. nNOS	iNOS vs. eNOS	nNOS vs. eNOS
L-NAME	-	-	-
L-NIL	*	*	-
Aminoguanidine	-	*	-
7-NI	-	-	-
1400 W	*	**	**

*Selectivity: - non-selective, * partially selective, ** highly selective.*

Additionally to their influence on NOS, the NOS-inhibitors may have a wide range of known and/or unknown other effects with influence on other enzymes and biochemical reactions, which has to be considered when they are used *in vivo* (Alderton et al., 2001, Southan and Szabo, 1996).

L-NAME and L-NIL

In order to limit NO production, two different NOS inhibitors were used; the non-selective N^G -nitro-L-arginine methyl ester (L-NAME) (III) and the partially iNOS-selective inhibitor L-*N*-iminoethyl-lysine (L-NIL) (IV). As mentioned above, the arginine analogue L-NAME is a unselective, competitive NOS-inhibitor acting on the substrate-binding site of the NOS enzyme (Moncada et al., 1991) and has a higher affinity for the constitutive isoforms eNOS and nNOS than iNOS (Southan and Szabo, 1996). L-NAME has been widely used as a pharmacological modulator of NOS activity after experimental cerebral ischemia (Iadecola, 1997).

S-PBN

The free radical scavenger and nitrone spin trap agent, 2-sulfo-phenyl-N-*tert*-butyl nitrone (S-PBN), was used to scavenge ROS and thereby limit superoxide levels. S-PBN has recently been

shown to be an effective microcvascular ROS scavenger in this TBI model (Marklund et al., 2001c). S-PBN is a spin trapping agent that forms stable adducts with carbon and oxygen centred radicals and reduces salicylate hydroxylation (Carney and Floyd, 1991). Previous findings have indicated that the neuroprotective actions of S-PBN might be exerted by protection of the microvascular endothelium and the blood-endothelial interface (Marklund et al., 2001b, Marklund et al., 2001c). This hypothesis is further supported by the fact that the endothelial cells are important ROS generators (Kuroda et al., 1999, Wei et al., 1981), and that the cerebrovascular endothelium is exposed to neutrophils and platelets, which forms ROS (Kontos and Povlishock, 1986, Siesjo, 1992a, Siesjo, 1992b). S-PBN has poor BBB penetration due to its hydrophilic properties and may thereby have difficulties to scavenge ROS in the parenchymal cells in the brain. However, as described above, BBB breakdown is well known sequelae of experimental TBI and S-PBN may theoretically leak into and affect the brain parenchyma. In the present study, no attempt was made to confirm the scavenging effects of S-PBN by specific assessments, since they were recently documented in this experimental model of TBI (Marklund et al., 2001c).

STATISTICAL ANALYSES

Dunnets test was used in paper I. One-way analysis of variance (ANOVA) and Dunnetts test for comparison of multiple groups with one control group were used in paper III and V. Unpaired t-test with welch correction were used in paper IV. Gaussian distribution of the data was tested using the method of Kolmogorov and Smirnov in paper III-V. In the results section, all quoted comparisons using the term “increase” or “decrease,”reflect statistically significant, unless otherways stated.

RESULTS AND DISCUSSION

NOS EXPRESSION FOLLOWING BRAIN CONTUSION (I AND II)

Experimental contusions

We demonstrated increased immunoreactivity for all three NOS isoforms after trauma. The development of NOS production was followed for 48 hours after trauma. The number of eNOS-positive cells increased early with a significant difference at 6 hours post trauma, but became equal to sham-operated animals at later time-points. eNOS immunoreactivity was confined to cells that with endothelial morphology, which is similar to experimental cerebral ischemia and suggests that posttraumatic increases of NO derived from eNOS is an early event and mainly affects the cerebral vessels (Iadecola, 1997, Zhang Z. G. et al., 1993).

The number of nNOS-immunoreactive cells also exhibited an initial, non-significant increase compared to sham-operated animals, followed by a significant decrease at 48 hours post trauma. The findings agree with previous studies on experimental ischemia and TBI (Higuchi et al., 1996, Rao et al., 1999, Zhang Z. G. et al., 1994). The later decrease probably reflected loss of neurons and neuronal dysfunction resulting from neuronal death (McIntosh, 1994). The majority of cells positive for nNOS-immunoreactivity also expressed the neuronal cell marker NeuN, but some nNOS-positive cells instead expressed the marker for neutrophils (W3/13); Chen and Mehta (1996) have previously demonstrated nNOS synthesis in neutrophilic white blood cells.

iNOS immunoreactivity was not detected in sham-operated animals. A few iNOS-positive cells were detected at 6 hours post trauma, they increased with a significant peak at 24 hours post trauma, followed by a gradually decrease at 36 and 48 hours (Fig.9). The finding is similar to experimental ischemia and TBI in immature rats (Clark et al., 1996b, Iadecola et al., 1995a). The major cellular sources of iNOS were macrophages and neutrophils, immunoreactive for ED-1 or W3/13. However, at 24 hours post trauma, some iNOS-positive cells showed of neuron-like morphology and were co-labelled with the neuronal cell marker (NeuN). Petrov et al. had similar observations in experimental severe, diffuse TBI (Petrov et al., 2000). In previous studies, iNOS was induced also in endothelial cells (Petrov et al., 2000, Wada et al., 1998), but we found no endothelial iNOS-immunoreactivity in this trauma model for brain contusion. The temporal profile and cellular sources for iNOS suggested that iNOS was confined to the postcontusional inflammatory reaction (Holmin et al., 1995, Holmin et al., 1998), and is a likely pathophysiological mediator in the inflammatory brain damage also following experimental brain contusion; iNOS induction is a detrimental event in postischemic inflammation (Iadecola et al., 1995b, Iadecola et al., 1997).

Taken together, the findings indicated that NOS immunoreactivity increase during the first hours-days after experimental contusion, and that NO may be derived from the endothelial cells in the vessels, the parenchymal neurons and from the cells in postcontusional inflammatory reaction in this setting.

Human contusions

Very few studies describe the role of NO in patients suffering from TBI. Clark et al. (Clark et al., 1996a) found increases in cerebrospinal fluid nitrite and nitrate concentrations that peaked

at 30 to 42 hours after severe closed-head injury. The increase in cerebrospinal fluid nitrite and nitrate concentrations was greater in non-survivors, indicating that increased production of NO in the brain is associated with the extent of injury and death. The cellular sources and tissue compartments of NO produced in human brain tissue after TBI has not earlier been identified. The purpose of this study was to investigate whether the experimental findings could be reproduced in clinical TBI. Contused brain tissue biopsies from patients who were surgically treated at different time points after trauma, (3-120 hours post trauma) were studied. eNOS and nNOS immunoreactivity was observed in brain tissue from trauma patients as well as from controls (normal cortical brain tissue from human patients undergoing surgical treatment for epilepsy). In contrast to our experimental findings, traumatic changes could not be proven. However, possible smaller differences in eNOS and nNOS immunoreactivity, compared with baseline levels in control brain tissue, are difficult to detect because of the limited number of patients and the difficulty of quantification in surgical biopsies, since the antigen is expressed also under normal conditions. It cannot also be excluded that the power of our method was not sufficient to detect differences in eNOS and nNOS expression. The cellular sources of nNOS and eNOS-positive cells were identified as neurons and endothelial cells, respectively, which is in agreement with the experimental data. iNOS immunoreactivity was not detectable outside blood vessels in control specimens. In traumatized brain tissue, some iNOS immunoreactivity was detected already in patients operated within the first 6 hours. The number of iNOS-positive cells peaked in patients who were surgically treated between 8-23 hours after trauma, followed by a slight decrease in patients operated between 84-120 hours post trauma (Fig 9).

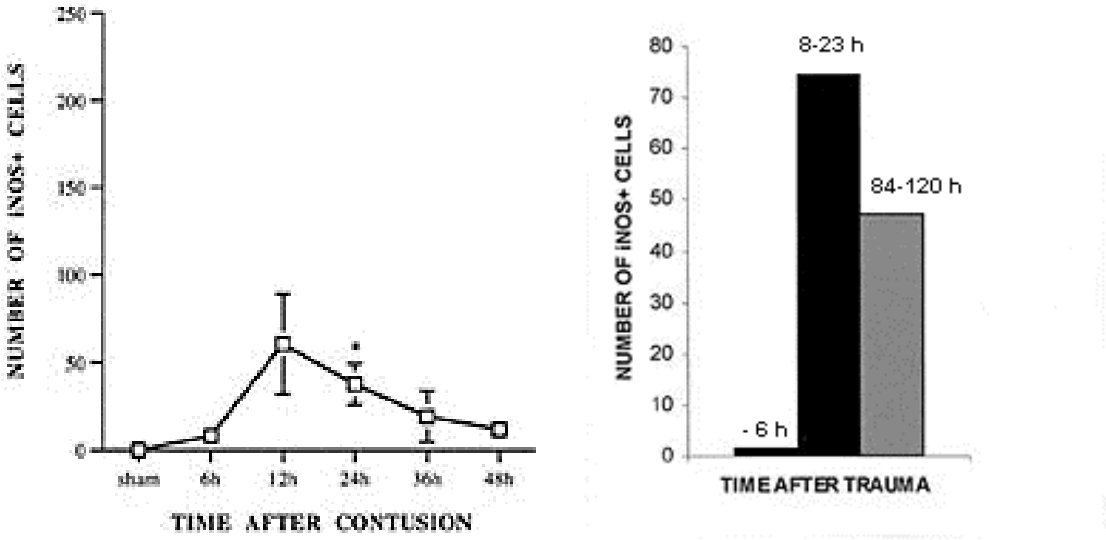

Figure 9. *Similar temporal profile for iNOS immunoreactivity was seen in rodents (left), and humans (right) after brain contusion. In both species, a few iNOS-positive cells were detected within the first 6 hours, followed by a marked increase with a peak around 24 hours after trauma, and then a decrease at later time-points.*

The main sources of iNOS-positive cells in the traumatized human brain were identified as neurons (NeuN), macrophages (ED-1), and neutrophils (W3/13), which is in agreement with the experimental data and other experimental findings (Petrov et al., 2000, Wada et al., 1998).

A few iNOS-positive cells were co-labelled with astrocytic cell marker (GFAP) and cell marker for oligodendrocytes (Transferrin). The ratio and intensity of staining in iNOS-positive neurons and leukocytes seemed to differ in samples taken early an late after trauma, Patients surgically treated 8 or 12 hours after trauma, showed an infiltration of strongly iNOS-positive cells that exhibited morphology of macrophages and neutrophils (67% of iNOS-positive cells), whereas in patients operated 23, 84 or 120 hours after trauma, weakly stained cells were found that, in contrast to our experimental findings, mainly demonstrated morphological features of neurons (85% of iNOS-positive cells). In contrast to the present experimental findings, Petrov et al used the Marmarou TBI model, which causes extensive diffuse brain injury and observed a delayed induction of iNOS protein in neurons (Petrov et al., 2000). Thus, the cellular sources of iNOS, agreed with data on severe experimental TBI. Also, the human brain tissue samples we used in this study were obtained from patients with severe injuries, for whom surgery was performed to prevent death. It seems possible that the severity of brain injury influences the neuronal expression of iNOS. However, a large group of patients with brain contusions have low-energy trauma and present in more favourable clinical conditions, in which surgery is not needed unless a delayed detorioration occurs (Mathiesen et al., 1995). Such patients probably have injuries with more similarities to the experimental brain contusions produced in our trauma model. For obvious reasons, it is not possible to analyze brain tissue from mildly injured patients to compare the cellular sources of iNOS. It is also possible that the cellular sources of NOS expression following TBI are different for different species.

To summarize, human data and previous experimental reports indicate that the spatial and temporal patterns of nNOS and iNOS expression in discrete cellular populations appears to be complex, indicating different time-points of NO synthesis following TBI, depending on the injury mechanism and the severity of injury. However, the human data largely parallel experimental findings and indicate that such trauma models are relevant for experimental studies and pharmacological treatment.

EFFECTS OF NOS INHIBITORS (III AND IV)

One purpose of the present study was to further examine neuronal injury and cellular apoptosis, and its relation to peroxynitrite formation after experimental brain contusion. As mentioned in the introduction, peroxynitrite ($ONOO^-$) is synthesized from NO and superoxide anion ($O_2^{\cdot-}$). We also wanted to investigate the effects on histopathological damage when one or both of the substrates for peroxynitrite are limited by pharmacological treatment. Histopathological damage was estimated at 24 hours and 6 days post trauma, by assessing neuronal degeneration and survival, and cellular apoptosis in the injured hemisphere. Peroxynitrite formation was estimated indirectly by immunoreactivity for nitrotyrosine (NT) at 24 hours post trauma. The time-points in these studies (III, IV) were set to 24 hours post trauma, to detect early posttraumatic effects of treatment, and to 6 days post trauma to include the peak of parenchymal inflammation (Holmin et al., 1995), which may be a significant source of ROS and iNOS derived NO after injury.

Non-selective NOS inhibition (III)

Treatment with L-NAME (III) significantly reduced the levels of citrulline derived from all NOS isoforms in the traumatized hemisphere, but slight indicatively more effectively on calcium-dependent NOS (eNOS/nNOS) (-80.0%) than for calcium independent NOS (iNOS) (-78.9%, non-significant). The mean value for iNOS activity was clearly reduced but did not reach statistical significance in this sample size, possibly due to the large variations of iNOS activity in the controls. The finding is, however, in agreement with the described action of L-NAME (Moncada et al., 1991), with a slight higher affinity for the constitutive isoforms eNOS and nNOS than iNOS (Southan and Szabo, 1996). The immunoreactivity of iNOS was unaffected, indicating that L-NAME did not affect iNOS mRNA expression, which corresponds to the view that L-NAME limits iNOS activity due to substrate competition with arginine rather than on the transcriptional level (Singh et al., 1996, Southan and Szabo, 1996).

L-NAME-treatment significantly reduced neuronal degeneration (-40.7 %) and cellular apoptosis (-32 %) at 24 hours post trauma, but with no persistent effect at 6 days. However, the number of surviving neurons in the perilesional cortex was increased at both time-points (+19.0% and +35.7%, respectively) (Fig.10), which indicated a total neuroprotective effect by L-NAME-treatment.

NOS inhibition in combination with a ROS-scavenger (III)

The combined treatment with L-NAME and S-PBN similarly reduced NOS-activity of eNOS/nNOS (-71.0%, significant) and iNOS (-78 %, non-significant), in comparison with L-NAME alone. However, administration of only the ROS scavenger and nitrone spin trap agent S-PBN, did not separately affect iNOS or eNOS/nNOS activity (-6.9%, non-significant and +9.8%, non-significant, respectively), nor did it influence iNOS immunoreactivity. This indicated that S-PBN, in contrast to PBN (Samuelsson et al. 2003) was not an efficient NOS-inhibitor. Accordingly, the NOS-activity inhibition demonstrated by the combined treatment was related to L-NAME.

Single S-PBN-treatment significantly reduced the number of degenerating neurons at 24 hours (- 38.8 %), and significantly increased the number of perilesional residual cortical neurons at 6 days (+52.1 %) post trauma (Fig.10), which indicated a neuroprotective effect of S-PBN, and corroborated recent findings on experimental TBI (Marklund et al., 2001b). The combination of L-NAME and S-PBN also decreased neuronal degeneration at 24 hours (- 34.7 %) and increased neuronal survival (residual perilesional cortical neurons) at 6 days (+ 47.7 %) (Fig.10). In contrast to the single agent administration, the combined treatment significantly reduced neuronal degeneration also at 6 days post trauma, which may indicate a more prolonged effect on this histopathological parameter. However, combined treatment showed no difference in neuronal degeneration, cellular apoptosis or neuronal survival, compared to single agent administration, which indicated that the combination offered no further neuroprotection. This finding was unexpected, since we assumed that both drugs have other mechanisms of action than limiting NT/peroxynitrite formation. The findings indicated a dominant pathogenic role for peroxynitrite formation in experimental contusion.

Nitrotyrosine

Nitrotyrosine (NT) immunoreactivity was significantly reduced following single administration of L-NAME (-62 %), which corroborated earlier findings in mice (Mesenge et al., 1998), and

indicated that the protein nitration caused by NO-derived species, e.g. peroxynitrite, was reduced. It was also found that S-PBN alone reduced NT immunoreactivity (-68 %); the finding was novel but not surprising, since another nitrone spin trap, PBN, had this effect in experimental colitis (Naito et al., 2002). The combined treatment with L-NAME and S-PBN also reduced the NT-immunoreactivity significantly (-70 %), but no additive effect compared to single agent administration was found.

In the present study, the absent additive effect by the combination of L-NAME and S-PBN on NT formation may to some extent be expected, since both compounds reduced NT formation, and a lack of substrate, i.e. NO- and ROS-derived species and reactions, from either side of the pathway may thus possibly give the same reduction of protein nitration.

NT was detected in activated microglia, infiltrating neutrophils, neurons and astrocytes, which is in agreement with previous findings regarding NT immunoreactivity (Catania et al., 2003, Forster et al., 1999, Hall et al., 1999). NT-immunoreactivity was not present only in the invading inflammatory cells, but also in neurons, where co- expression of NT and nNOS was detected; hence protein nitration occured in close vicinity of the neuronal sources of NO. These findings indicated that peroxynitrite acted directly in the nNOS-positive neurons, but also diffused to affect adjacent neurons by a bystander effect. The proportions of neurons among all NT-positive cells was slightly lower in animals treated with L-NAME (7 %) or the combination of L-NAME and S-PBN (10%) than in controls (16 %), which indicated that inhibition of NOS in the neurons resulted in less tyrosine nitration caused by peroxynitrite and/or NO-related nitrosative stress.

Selective iNOS inhibition (IV)

In paper IV, the role of iNOS derived NO on secondary cellular injury and peroxynitrite formation was further studied.

Posttraumatic treatment with the partially iNOS-selective inhibitor L-NIL significantly reduced the enzymatic activity for iNOS in the injured area (-59.1%) compared to controls. No significant change of eNOS/nNOS activity was observed. This finding further supported that NOS can be inhibited by pharmacological treatment, and also supported the postulated iNOS-selectivity for L-NIL (Moore et al., 1994). Moreover, iNOS immunoreactivity was unchanged after L-NIL treatment, which agrees with the suggestion that L-NIL inactivates the enzyme by altering the functionality of the active site of iNOS (Bryk and Wolff, 1998).

L-NIL treatment reduced the number of degenerating neurons at 24 hours (-42.9 %), which is in agreement with recent findings with the partially selective iNOS inhibitor aminoguanidine (AG) in lateral fluid percussion-TBI (Lu et al., 2003b). The amount of neuronal apoptosis (the proportion of morphologically typical TUNEL-positive cells co-labelled with NeuN) was not significantly different between the groups, which indicated that L-NIL treatment mainly reduced neuronal necrosis at 24 hours. A possible explanation to this finding may be that inhibition of iNOS by L-NIL could limit peroxynitrite-induced activation of PARP (and subsequent energy depletion), and thus reduce neuronal necrosis. It is known that apoptosis requires energy and that intracellular energy levels may regulate whether a cell undergoes necrosis or apoptosis, i.e. energy depletion results in necrotic cell death (Eguchi et al., 1997). The number of residual perilesional cortical neurons was unchanged by L-NIL, which indicated that iNOS-derived NO affected the degenerative processes in neurons still expressing NeuN at 24 hours post trauma. The number of degenerating neurons was not significantly affected at 6 days post trauma whereas neuronal survival was increased following L-NIL-treatment (+59.2 %) at this time-point (Fig.10). This indicated that the decreased neuronal degeneration

(apoptosis/necrosis) by L-NIL in this setting was an early event resulting in attenuated neuronal loss at 6 days. The reduction of neuronal degeneration at day 6 (-21.2%) was non-significant in this sample size. Total cellular apoptosis (TUNEL + morphological criteria) was unchanged at 24 hours, but significantly decreased at 6 days (-25.6 %), which indicated that iNOS-derived NO affected cellular apoptosis whith a delay after trauma. The relative proportion of apoptotic cells that co-expressed ED-1 or NeuN at 6 days was equal in both groups, which indicated that L-NIL treatment reduced apoptosis also in macrophages, which is in agreement with recent findings by Lu et al. (Lu et al., 2003a, Lu et al., 2003b). This delayed effect of L-NIL treatment on cellular apoptosis correlates with the maximal intracerebral inflammatory response after TBI (Holmin et al., 1995). Furthermore, Lu et al. found that NO is also linked to elimination of brain macrophages by apoptosis (Lu et al., 2003a). Additionally, we found that inflammatory cells are a major source of iNOS-immunoreactivity in this model of TBI (I). We demonstrated a marked reduction of NT-immunoreactivity by L-NIL-treatment (-53.6 % p = 0.0007 unpaired t-test with welch correction), which together with other data indicated that the neuroprotective effect of L-NIL was mediated, by a reduction of detrimental peroxynitrite related protein nitration.

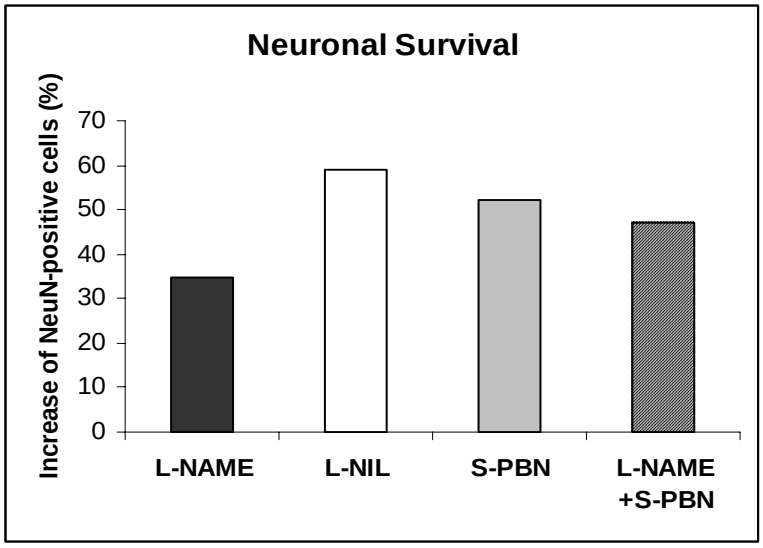

Figure 10. *The number of residual NeuN-positive neurons in the perilesional cortex was increased (%), following post-traumatic treatment with L-NAME (non-selective), L-NIL (iNOS-selective), S-PBN (ROS-scavenger), or a combined treatment with L-NAME and S-PBN, compared to controls at 6 days post trauma.*

In summary our findings suggested an over all neuroprotective effect of posttraumatic treatment with unselective or iNOS-selective NOS-inhibition during the first days post trauma in this model of experimental contusion; the conclusion corroborates previous findings (Jafarian-Tehrani et al., 2005, Mesenge et al., 1996, Sinz et al., 1999, Stoffel et al., 2001, Wada et al., 1998, Wada et al., 1999).

NEUROPROTECTIVE DRUGS WITH AN INDIRECT EFFECT ON iNOS (V)

There are several drugs with different neuroprotective actions that also may affect iNOS transcription. Dexamethasone, Tirilazad Mesylate and Nimodipine are drugs that target different mechanisms of secondary injury following TBI. All drugs have been neuroprotective in experimental models of TBI and ischemia, yet no pharmacological strategy has shown

potential for a general reduction of secondary injury in clinical trials (Narayan et al., 2002). Colchicine is a drug with anti-inflammatory properties (Malawista, 1968, Molad, 2002) that has been demonstrated to reduce inflammatory response and oedema development in the TBI-model used in the present study (Holmin and Mathiesen, 1996). Paper V describes the effects on histopathological damage and iNOS immunoreactivity following posttraumatic treatment with Colchicine, Dexamethasone, Tirilazad Mesylate or Nimodipine. The drugs were administrated 4 hours post trauma, a time point chosen because it reflects a feasible time to start pharmacological treatment in a clinical scenario.

Colchicine

Colchicine is an anti-inflammatory drug that modulates chemokine and prostanoid production, inhibits neutrophils, macrophages and endothelial cell adhesion molecules and thereby interferes with the inflammatory reaction (Holmin and Mathiesen, 1996, Molad, 2002). In colchicine-treated animals, a significant decrease of degenerating neurons in the traumatized area at 24 hours (Fig. 11), together with a significant increase of residual neurons in the perilesional cortex at 14 days post trauma were found (Fig. 12). The proportion of TUNEL positive cells that co-expressed neuronal cell marker, of all TUNEL-positive cells, was also slightly reduced in at 24 hours compared to controls (*colchicine;* 31.2%, *controls;* 53.2%) At later time points, no differences in FJ- or TUNEL-reactivity were detected, which indicated that Colchicine mainly affected degeneration during the first day/days after trauma. The findings indicated a protective effect for Colchicine, when administrated 4 hours post trauma. Colchicine is protective in ischemic lesions (Giulian and Robertson, 1990, Giulian et al., 1993) and following TBI (Holmin and Mathiesen, 1996). The finding of a reduced neuronal degeneration in experimental brain contusion was new and suggested that anti-inflammatory therapies targeted to macrophages/microglial activation may be beneficial for contusional injuries with a moderate energy transfer. The protective effect appeared to be specific for neurons, since TUNEL-staining of inflammatory cells and glia was unaffected,

Dexamethasone

Dexamethasone is a corticosteroid; corticosteroids have anti-inflammatory effects via intracellular receptors and inhibit virtually all cellular- and humoral inflammatory functions. They also have an antioxidant potential through inhibition of lipid peroxidation in CNS injury (Anderson et al., 1985). A significantly reduced number of FJ-positive cells at 24 hours post trauma were seen following posttraumatic treatment with Dexamethasone (Fig. 11). The total number of TUNEL-positive cells was unchanged but the proportion of TUNEL-positive cells that co expressed NeuN was lower than in controls at 24 hours (*Dexamethasone;* 26.6%, *controls*; 53.2%). These findings indicated that Dexamethasone had an early neuroprotective effect without affecting other cell-populations. However, at 5 and 14 days, the number of FJ-positive cells was significantly higher than in controls (Fig. 11), and the total number of residual neurons in the perilesional was not different from controls at 14 post trauma (Fig. 12). Two explanations are possible: Dexamethasone treatment may have delayed the post-traumatic inflammatory reaction and retarded neuronal degeneration. Different mechanisms of neuronal degeneration appear to be relevant at different times after trauma for this model of brain contusion (Wennersten et al., 2003). Another possibility is that the prolonged Dexamethasone treatment had direct neurodegenerative effects. Corticosteroids in high doses may induce neuronal apoptosis in the CNS (Haynes et al., 2001, Mitchell et al., 1998). The staining pattern

of albumin immunoreactivity showed albumin extravasation at day 5 in Dexamethasone-treated animals, which indicated an improved BBB function. Immediate damage to the brain parenchymal blood vessels may be directly produced by the trauma and obscure a possible early drug effect on membrane stability. Dexamethasone appeared to reduce the post-traumatic vasogenic brain oedema, which is in agreement with other studies by Holmin and Mathiesen (1996) and Hortobagyi et al (2000). The effect on albumin extravasation could be direct membrane-stabilization or mediated by its anti-inflammatory properties**.**

Taken together, our findings indicated both beneficial and detrimental effects of prolonged posttraumatic Dexamethasone treatment, which fits with previous conflicting studies showing that neither moderate benefits nor moderate harmful effects of steroids can be excluded (Alderson and Roberts, 2000). Experimental studies have shown that corticosteroids are protective following subarachnoid haemorrhage (SAH) and brain trauma (Hall, 1985, Hall and Travis, 1988) while clinical studies have failed to detect a general neuroprotective effect (Alderson et al., 2000, Alderson et al., 2005, Narayan et al., 2002). This is to our knowledge the first experimental analysis of Dexamethasone treatment and neuronal cell death in a rodent model of brain contusion. An early effect on neurodegeneration seemed clear. This model mimics brain contusion (Feeney et al., 1981) and a multicenter study (Grumme et al., 1995) showed a protective effect of corticosteroids on patients with brain contusions when prolonged treatment was used. It is possible that corticosteroids in the future may prove clinically useful for certain subgroups of TBI patients.

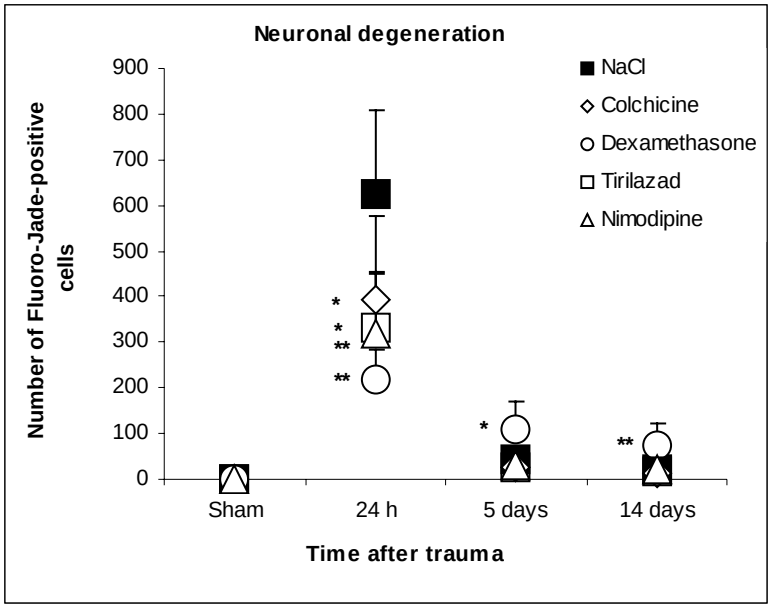

Figure 11. *Expression of Fluoro-Jade (FJ) at 24 hours, 5 and 14 days after an experimental brain contusion.* ***A****: FJ expression was significantly decreased in all treatment groups at 24 h compared to controls (NaCl). At 5 and 14 days, a reduction of FJ expression over time was seen in all groups, with a delayed manner in Dexamethasone treated animals. * = p<0.05; ** = p<0.01.*

Tirilazad Mesylate

Tirilazad Mesylate is a lazaroid (Kavanagh and Kam, 2001). The major site of action for Tirilazad Mesylate appears to be the BBB. It is localized in the cerebrovascular endothelium and numerous studies show an attenuation of injury, and ischemia-induced BBB-permeability

(Hall, 1995, Kavanagh and Kam, 2001). Tirilazad Mesylate has neuroprotective efficacy in multiple preclinical models of TBI, SAH (subarachnoidal haemorrhage), and focal cerebral ischemia (Hall, 1995, Kavanagh and Kam, 2001). It decreases cerebral vasospasm, BBB compromise, post-traumatic ischemia, oedema, ischemic neuronal necrosis and infarction, and improves neurological recovery (Hall, 1995, Kavanagh and Kam, 2001). These effects are correlated with a reduction in markers of oxygen radical-induced lipid peroxidation (Hall, 1995, Kavanagh and Kam, 2001). Lazaroids inhibit lipid membrane peroxidation via free radical scavenging and membrane stabilization (Kavanagh and Kam, 2001). We found that Tirilazad Mesylate significantly decreased the number of degenerating neurons (Fig 11) and reduced the relative proportion of NeuN/TUNEL-positive cells at 24 hours (*TM;* 28.4% *controls;* 53.2%). In addition, a significant increase in neuronal survival was seen at 14 days (Fig.12). The findings indicated a neuroprotective effect in this kind of contusional injury. Surprisingly, the total number of TUNEL-positive cells was increased at 5 days post trauma. At 5 and 14 days, the majority of TUNEL-positive cells co-expressed non-neuronal cell markers. Tirilazad Mesylate thus spared neurons but increased cellular death in the pool of cells encompassing leukocytes and macrophages. An antioxidant effect of Tirilazad Mesylate, resulting in lower amounts of free radicals causing less DNA damage and neurotoxicity was possible; the latter finding was compatible with an overall anti-inflammatory effect.

Nimodipine

The calcium-channel blocker Nimodipine acts on the L-type of voltage gated calcium channel in the neuronal membrane (Langham et al., 2000). Nimodipine has become standard treatment for patients with nontraumatic subarachnoid haemorrhage. In clinical traumatic brain injury, nimodipine has shown to be beneficial in younger patients and in patients with posttraumatic subarachnoid haemorrhage (Harders et al., 1996, Langham et al., 2000), although this conclusion is debated since several trials of nimodipine treatment in human head injury were negative regarding their primary endpoints (Murray et al., 1996, Narayan et al., 2002, Teasdale et al., 1990). Interestingly, subgroup analysis of clinical trials suggested a better outcome with nimodipine treatment in the patients with intra-axial contusions without extra cerebral haematomas (Mathiesen et al., 1995). The effect of nimodipine treatment has shown contradictory results in experimental TBI (Ercan et al., 2001, Ustun et al., 2001). We found a significant early decrease of degenerated neurons, both Fluoro-Jade positive cells (Fig. 11), and the proportion NeuN-positive/TUNEL-cells (*nimodipine;* 35.4% *controls;* 53.2%) decreased following Nimodipine treatment. The findings support a possible neuroprotective posttraumatic effect. However, a significant difference did not remain, and neuronal survival nor affected at day 14 (Fig. 12).

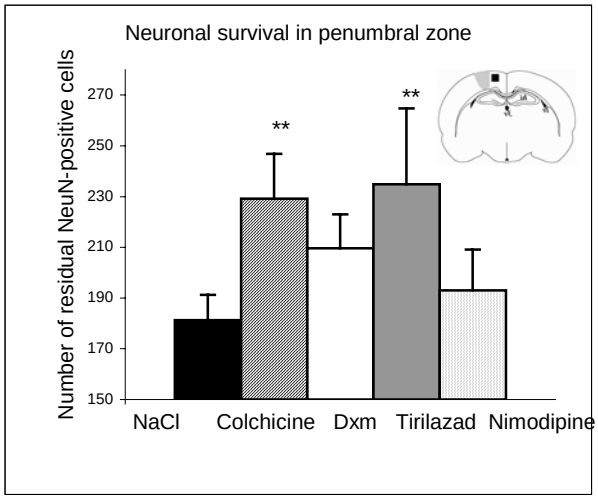

Figure 12. *Number of NeuN-positive cells in the penumbral zone 14 days after trauma. Black box represents analysed area in the perilesional area, including the penumbral zone. The number of NeuN-positive residual cells was significantly increased in animals treated with Colchicine or Tirilazad mesylate. ** = p<0.01.*

Effect on iNOS expression

A significant decrease of iNOS positive cells in animals treated with Colchicine, Tirilazad Mesylate or Nimodipine was demonstrated at 24 hours post trauma (Fig. 13). At later time points (5 and 14 days) no significant differences of iNOS immunoreactivity were seen between treatment groups and controls. The drugs had the most potent effect on iNOS induction early after injury but the number of iNOS-positive cells was low at the later time-points also in controls. The data are compatible with a hypothesis that iNOS may be a common pathway for early neuronal injury in this model. The effect on iNOS expression was similar in Colchicine, Tirilazad Mesylate and Nimodipine groups, although they have different mechanisms of action and despite the fact that their neuroprotective properties are presumably linked to mechanisms other than iNOS expression. The early decrease in iNOS expression that was detected is compatible with a drug effect on the iNOSmRNA expression. All drugs have a potential to affect iNOS transcription: Dexamethasone may decrease iNOS mRNA/protein expression by inhibition of NFkB (Ganster and Geller, 2000), and Colchicine may decrease iNOS mRNA/protein expression by depolymerization of microtubules (Marczin et al., 1996). Additionally, Tirilazad Mesylate like other antioxidants may reduce iNOS transcription (Salahudeen et al., 1996). The suppression of iNOS immunoreactivity by Nimodipine was a new finding, but Zhu et al. have earlier demonstrated a reduced infarct size, associated with a reduced activity of the iNOS enzyme, following Nimodipine treatment in experimental focal cerebral ischemia (Zhu et al., 1999). It has been suggested that Nimodipine also may act on the transcriptional level of iNOS (Cattaruzza et al., 2000), which agrees with our finding that Nimodipine reduced the immunoreactivity for iNOS already at 24 hours. Since NFkB is a critical transcription factor for iNOS expression (Ganster and Geller, 2000), and since activation of NFkB may be caused by both proinflammatory cytokines (e.g. TNF-α and IL-1β) and ROS, another possibility for the reduced iNOS induction by these four drugs would be that the drugs limited secondary neurodegeneration via their intended mechanisms (anti-inflammatory, ROS-scavenging, calcium channel-blockage), and that decreased cellular injury reduced secondary iNOS synthesis.

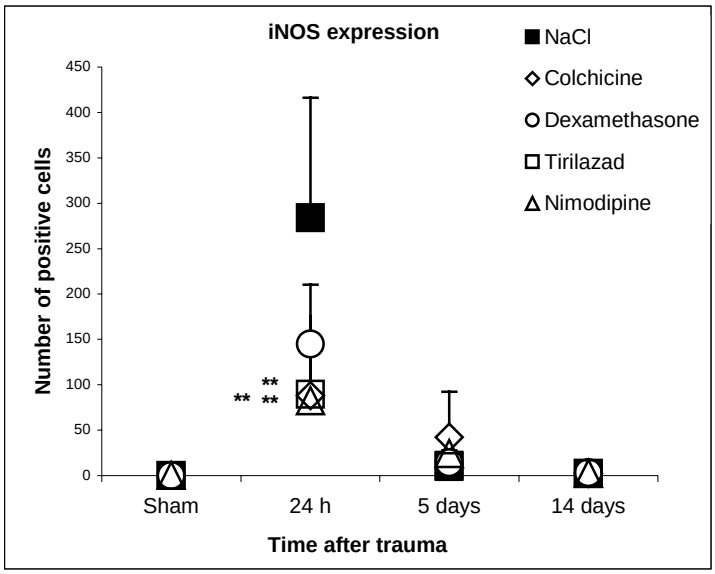

Figure 13. *iNOS expression was decreased in all treatment groups at 24 h (significant for Colchicine, Tirilazad Mesylate and Nimodipine). No significant difference was seen at 5 and 14 days. ** = p<0.01*

To summarize, the findings demonstrated that posttraumatic treatment with Dexamethasone, Colchicine, Tirilazad Mesylate or Nimodipine reduced early neuronal degeneration in this trauma model for focal brain contusion. The findings were based on adequate drug doses, but additional titration of dosage, although laborious, may refine data such as these. In this setting, all drugs had some neuroprotective properties and all attenuated early iNOS-synthesis, but only Colchicine and Tirilazad Mesylate enhanced final neuronal survival. These findings would support that neurotoxicity after low/moderate energy contusion involves parenchymal inflammation, increased levels of free radicals and intracellular Ca^{2+} and that NO derivable from iNOS is a possible common pathway. A next step would be to combine different drugs as was done in (III) in order to sort out the extent of common pathways, and potential to improve outcome be simultaneous targeting of different pathophysiological mechanisms.

GENERAL DISCUSSION

The findings in this thesis corroborate the hypothesis that NO production plays an important role in the development of secondary injury following TBI.

We demonstrated that immunoreactivities for the constitutive NOS isoforms, eNOS and nNOS, were increased during the first hours after trauma in endothelial cells and neurons, respectively. The non-constitutive, inducible NOS isoform, iNOS, was induced mainly in inflammatory cells with a peak one day after trauma. Brain biopsies were examined from patients undergoing surgery for cerebral contusions, and we found that iNOS was induced also following human contusion. The temporal pattern agreed with the experimental findings. The cellular sources of iNOS following human contusion differed at different times after trauma. During the first twelve hours, the major source was inflammatory cells, whereas neurons dominated between one and three days after trauma. iNOS in the early phase can thus be viewed as part of the inflammatory reaction, while neuronal iNOS is a result of gene activation, probably through NFκB pathway by e.g. TNF-α or IL-1β. Since the findings in humans and rodents were similar, the trauma model was considered relevant for experimental studies and treatment trials.

It was demonstrated that posttraumatic treatment with a NOS-inhibitor (L-NAME), a selective iNOS-inhibitor (L-NIL), and the free radical scavenger (S-PBN) were neuroprotective and reduced histopathological damage at 24 hours and 6 days post trauma. All three drugs reduced NT-immunoreactivity, an indirect marker for peroxynitrite fromation. Surprisingly, the combination of a NOS-inhibitor and a free radical scavenger was not additionally neuroprotective. The findings support that NO produced during the first days is detrimental to the injured brain and that an important pathway of NO-mediated toxicity is the formation of peroxynitrite. Moreover, the absence of an additive effect suggests that peroxynitrite formation and protein nitration is of dominating importance in this setting.

Posttraumatic treatment with four different drugs, each with a different mechanism of action, directed against inflammatory-, free radical- or calcium-mediated toxicity, reduced histopathological damage early after trauma, but also reduced the immunoreactivity for iNOS. Their influence on iNOS may be either direct on the transcription factors that mediate iNOS expression, and/or indirect by limiting mediators that activate the same transcription factors; taken together, the findings support the role of iNOS as an early mediator of secondary neuronal damage.

Under pathological conditions, NO has several neurotoxic effects. NO also regulates several phisiological functions in the normal brain tissue, and has protective antioxidant effects; hence the consequences of NO-depletion by NOS-inhibition following TBI may be divergent. The impact of NO synthesis on different biological cascades can change rapidly, depending on the rate of NO formation and composition of the surrounding milieu (Radi et al. 2000). The chemical biology of NO may be divided into two major categories: direct and indirect. The direct NO-effects are suggested to be dominant under low NO concentrations, and the indirect effects by RNOS (N_2O_3 and peroxynitrite) dominate at high concentrations of NO (Wink and Mitchell, 1998); the result of the indirect effects, i.e. the protective effects by N_2O_3 and the detrimental effects by peroxynitrite, may probably be most important following a situation where NO production is increased, such as in the pathophysiology of TBI. An exception to this assumption might be the direct NO-effect on the cerebral vessels from eNOS in the endothelial cells and nNOS in the perivascular neurons.

Under aerobic conditions, when the generation of O_2^- is relatively low, and when there is an excess of NO over O_2^-, the formation of N_2O_3 is favoured (Espey et al. 2002). In contrast, when the levels of O_2^- are high, as in anaerobic conditions or in xantineoxidase rich cells, when NO at he same time is not in great excess, the formation of peroxynitrite is favoured (Espey et al. 2002). The inflammatory reaction following TBI involves xantine oxidase deposition and leucocytic infiltration under pathological conditions (Halliwell and Gutteridge, 1986, Matsuo et al., 1995). Under such conditions, higher rates of O_2^- and NO formation may coincide to generate a greater magnitude and duration of nitrosative and oxidative stress. In addition to the amount of O_2^- that is generated, the scavenge-velocity of O_2^- by Mn superoxide dismutase (MnSOD) will determine the ratio between NO and O_2^-, and thus indirectly whether the dominant NO derivate will be N_2O_3 or peroxynitrite, with its protective or detrimental effects. Peroxynitrite inactivates MnSOD (Radi et al. 2000), which may push the reaction towards peroxynitrite formation and its detrimental effects via NO/O_2^- production in the inflammatory reaction.

The N_2O_3-related protective or peroxynitrite-related detrimental effects provide one explanation for the contradictory findings of NOS-inhibition following experimental cerebral ischemia and TBI: Inhibition of NOS at a time when there is a need of increased perfusion, by NO-mediated vasodilatation, or an NO excess to favour N_2O_3 formation and protective nitrosation, would be detrimental.

It is known that NO-mediated vasodilatation is an important protective function in the early stages after cerebral ischemia and TBI, and too early NOS-inhibition is therefore detrimental (Iadecola, 1997, Wada et al., 1999). This might be explained in two ways: 1. Increased CBF, with increased perfusion of the ischemic brain parenchyma, promotes scavenging of toxic metabolites and reduces pathological anaerobic cellular metabolism. Increased NO production from eNOS in endothelial cells and nNOS in perivascular neurons will then result in a vasodilatation and a beneficial effect to the damaged brain. 2. Like in the pathophysiology that follows a subarachnoid haemorrhage, there might a relative deficit of vascular NO due to scavenging of NO by oxyhemoglobin also after a TBI (Ignarro, 1990, Wink and Mitchell, 1998). Hence, early eNOS/nNOS-derived NO production has the potential to restore NO-levels, and be protective. Consequently, treatment with NOS-inhibitors will probably not be of interest the first minutes/hour after trauma. In the used TBI-experimental model, NOS-inhibition was consequently initiated as earliest 15 minutes after the trauma.

However, iNOS-inhibition that is initiated after the period of beneficial NO-mediated cerebrovascular effect, and directed against the inflammatory reaction (between 24 hours and 4-5 days post trauma), may well have a beneficial total effect. iNOS is upregulated during the inflammatory response, and would be the prime target for such an approach. It has clearly been shown in this study that iNOS inhibition during the inflammatory phase induces a neuroprotection and thereby giving support to this hypothesis.

Furthermore, a prolonged posttraumatic elevation of intracellular calcium levels in neurons may keep the calcium-calmodulin-dependent nNOS enzyme active and subsequently perpetuate NO production. As a result, activated nNOS in neurons and iNOS in leucocytes may consume large amounts of L-arginine, which may lead to depletion of both intracellular-, and extracellular L-arginine in the vicinity of the nNOS/iNOS-expressing cells, which thus makes nNOS and iNOS responsible of both NO and O_2- production (Xia et al., 1996, Xia and Zweier, 1997) and secondarily promoted peroxynitrite-mediated neuronal damage following TBI.

Sinz et al. demonstrated that prolonged iNOS-inhibition was detrimental (Sinz et al. 1999), whereas we, in corroboration with several others (Jafarian-Tehrani et al. 2005, Mesenge et al., 1996, Wada et al., 1998, Wada et al., 1999) found neuroprotective effects of iNOS-inhibition when measured from 24 hours to 6 days post trauma. A possible explanation for the divergent results would be that pharmacological inhibition of iNOS might be beneficial and neuroprotective only when administered during the time of the inflammatory reaction, and that a more prolonged inhibition again will interfere with the protective NO-related effects.

The contradictory results of NOS-inhibition in experimental TBI may also be explained by the fact that NO-toxicity or -protection may depend on the type and degree of injury: different experimental TBI-models will then be affected differently by treatment with NOS-inhibitors. Several studies have demonstrated that the pathophysiology for secondary injury mechanisms may differ between different types of injury (Narayan et al., 2002), e.g. Nimodipine was protective only in the subgroup of patients with traumatic subarachnoidal haemorrhage (Harders et al., 1996), and delayed deterioration occured after contusions but not after traumatic intracerebral haematomas (Mathiesen et al., 1995). Additionally, in the clinical situation, it is not uncommon that a TBI-patient has a combination of vascular, parenchymal, focal and diffuse injuries. The heterogeneity of human TBI pathology and pathophysiology contributes to the difficulty to find an effective pharmacological treatment. There has been a consensus that a pharmacological treatment with a drug needs to have a robust effect in several different animal models if likely to work clinically (Narayan et al., 2002). In addition to our findings using the weight-drop model, NOS-inhibitors have shown beneficial effects in lateral fluid percussion-, cryogenic injury-models, and in a model for severe to moderate closed head injury, (Mesenge et al., 1996, Stoffel et al., 2000, Wada et al., 1998, Wada et al., 1999), whereas the effects of NOS-inhibitor are not much investigated in models for traumatic subarachnoidal haemorrhage.

The demonstrated neuroprotection by posttraumatic treatment with NOS-inhibitors after a focal cortical impact in a model for brain contusion could hardly serve as a pharmacological model for all types of TBI. In a clinical situation, our findings would more be applicable to patients who have contusions, without a combination of diffuse axonal injury, haematomas and shock. The model does not resemble a high-energy injury. The extent of neuroprotection was measured as reduced histopathological damage. It is generally accepted that reduced neuronal death is a prerequisite for neuroprotection but further experimental studies will be needed to clarify whether a histological neuroprotective effect also improves the behavioural and/or functional outcome. The TBI-model used in the present study produces a mild focal injury (Feeney et al., 1981), where the animals show no or minimal deficits post trauma, which make measurements of a behavioural/functional parameter difficult. Previous studies have, however, demonstrated improved functional outcomes 1-3 days post trauma, by NOS-inhibition in other types of experimental brain trauma (Lu et al., 2003b, Wada et al., 1999). It is likely that a functionally relevant effect (i.e. long-term) can be achieved with NOS-inhibitors during the first days post trauma although this has, to our knowledge, not yet been studied. Pharmacological treatment with selective nNOS and iNOS-inhibitors needs to be titrated regarding different modes of administration and end-points, and should include a long-term follow up. In addition to the reasons discussed above, there are other important factors that have to be further investigated and clarified, to rule out if a clinical trial with NOS-inhibitor treatment in TBI-patients could be recommended. Before any clinical trial is undertaken, a fundamental issue would be to analyze whether this treatment is experimentally effective in high-energy trauma, diffuse injuries and haematomas. If not, clinical trials need to include only patients with low-energy trauma and

contusions. Finally, it is also largely unknown how the critical window in a rodent model correlates with the window in humans. The effects of posttraumatic NOS-inhibition following TBI in more gyroencephalic species, such as monkey or pig, might be relevant to study, since those species reproduce human anatomy fairly well (Narayan et al., 2002).

Taken together, our data agree with a hypothesis that NO is an important pathogenic factor during postcontusional inflammation. Its selective inhibition would be expected to improve outcome at least following low-energy focal trauma, i.e. contusions. It appears worthwhile to pursue this hypothesis to clinical trials, since it regards a well-characterized traumatic mechanism, known to be relevant in a defined injury, and that can be interfered with in a controlled way that is not expected to inhibit physiologically valuable reactions. Another important continuation would be to examine other models to investigate whether a delayed NO-mediated and possibly inflammation-related neuronal degeneration is relevant also in DAI and vascular injuries.

CONCLUSIONS

The three isoforms of nitric oxide synthase (NOS) were upregulated after experimental brain contusion. The eNOS and nNOS isoforms were upregulated early with a maximum at 6-12 hours, whereas iNOS peaked later, at 24 hours after trauma. The immunoreactivity for eNOS and nNOS is predominantly detected in endothelial cells and neurons. iNOS was predominantly detected in inflammatory cells.

Immunoreactivity for iNOS increased also after human brain contusion. The cellular sources of iNOS differed at different times after trauma, inflammatory cells dominated the first hours, and neurons dominated from 23 hours post trauma.

Posttraumatic treatment with L-NAME reduced protein nitration and histopathological damage, indicating that NO and NO-derived species, such as peroxynitrite, contributed to secondary neuronal injury.

The combined treatment with L-NAME and the ROS scavenger S-PBN was neuroprotective but did not additively decrease apoptosis, neuronal degeneration or improved survival. Protein nitration, caused by e.g. peroxynitrite, occured both in invading inflammatory cells and in neurons

Posttraumatic treatment with a selective iNOS inhibitor reduced protein nitration and was neuroprotective, indicating that iNOS-derived NO production was detrimental rather than beneficial, and contributed to secondary neuronal injury during the first days post trauma.

Pharmacological strategies directed against inflammation, oxidative damage or calcium-overload had beneficial effects, during the first 24 hours; still all of them did not necessarily lead to increased long-term neuronal survival.

The ROS scavenger Tirilazad mesylate and the pro-inflammatory drug Colchicine were effective for amelioration of experimentally induced contusion.

Early neuroprotection by Colchicine, Dexamethasone, Tirilazad Mesylate and Nimodipine may additionally to their intended mechanisms of action also involve an influence on iNOS expression.

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♥

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